IMPROVEMENT OF INTENSIVE LARVAL REARING AND EVALUATION OF INLAND SALINE GROUNDWATER FOR AQUACULTURE OF SNAPPER, *Pagrus auratus*

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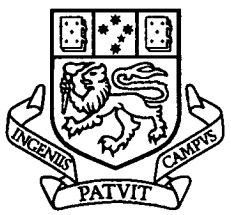

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DECLARATION AND AUTHORITY OF ACCESS

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ABSTRACT

This thesis identified optimal physical rearing regimes for Australian snapper, *Pagrus auratus* larvae, the suitability of saline groundwater for snapper culture, and the effects of salinity and potassium-deficient saline groundwater on osmoregulation of snapper.

The effects of photoperiod, salinity and temperature on growth, survival, onset of feeding, swimbladder inflation, presence of urinary calculi and tail flexion of first-feeding (3 days after hatching; dah) to pre-metamorphosis (21-32 dah) snapper larvae were determined in a series of factorial experiments conducted in specially designed, replicated, 100-l, cylindroconical tanks. The optimal photoperiod changed during larval ontogeny based on success of initial swimbladder inflation and subsequent growth. Snapper larvae tolerated a wide range of salinities from near-isoosmotic to hypersaline environments but optimal salinity for growth and development was from 20 to 35‰. Snapper larvae tolerated a relatively narrow range of water temperature from 15 to 24°C and larval growth increased as temperature was increased.

The performance of snapper larvae from 4-33 dah under a "new" regime that combined optimal salinity (20-35‰), temperature (24°C) and photoperiod (12L:12D to swimbladder inflation, then 18L:06D), determined systematically in a series of experiments, was compared with a previous "best-practice" regime of salinity (35‰), temperature (21°C) and photoperiod (14L:10D) in 2000-l commercial-scale larval rearing tanks. Larvae reared in the "new" regime grew and developed more quickly than larvae in the previous "best-practice" and by 33 dah were fully weaned from live feeds to a pellet diet. Approximately eleven hatchery cycles per year are possible when larvae are reared under the "new" regime compared with seven hatchery cycles per year for the previous "best-practice" regime.

The suitability of saline groundwater (SG; ~20‰) from inland New South Wales for growth and survival of juvenile snapper was determined in a series of replicated 7-8 d survival bioassays and 42 d growth studies in tanks of 2-l or 100-l, respectively. Raw SG was very deficient in potassium compared with similar salinity coastal seawater (CS) and snapper died within 2-3 d after transfer from CS to rawSG. However, growth and survival of snapper was the same in SG and CS, provided the $[K^+]$ in SG was increased to 60-100%

of the $[K^+]$ in CS by adding KCl. It was not possible to acclimate snapper to rawSG by dilution of $[K^+]$ over time.

The effects of rapid transfer of juvenile snapper from seawater (30‰) to near-isoosmotic (15‰) and hypersaline (45‰) seawater, and from seawater (30‰) to SG (30‰) fortified with 0 (rawSG), 40% (SG40) and 100% (SG100) of $[K^+]$ in seawater on serum osmolality, $[Na^+]$, $[K^+]$, $[Cl^-]$, blood haematocrit and branchial chloride cell morphology were assessed during 168 h after transfer. Changes in serum chemistry occurred rapidly in fish transferred to 15‰ and 45‰ but had returned to near initial levels after 168 h. Restoration of homeostasis was concomitant with changes in the number and size of immunoreactive chloride cells. Serum chemistry of fish transferred to SG40 and SG100 was similar, in general, to the initial levels. However, serum osmolality, $[Cl^-]$, $[Na^+]$ increased and serum $[K^+]$ decreased rapidly after transfer to rawSG. The morphology of chloride cells was unaffected by SG treatments.

The research described in this thesis has provided an experimental base for new culture conditions to greatly improve survival and growth of snapper larvae through to fingerlings and survival and growth of snapper juveniles in inland saline groundwater. These new culture conditions represent major cost savings to hatchery operations and improve the aquaculture potential of snapper in Australia.

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CHAPTER 1

General Introduction

1. General introduction

1.1. Aquaculture in Australia

The total production of world capture fisheries has remained relatively constant in the last decade and it is unlikely to increase as most stocks are fully or over exploited (FAO, 2000). In contrast, world aquaculture production of fish and shellfish is increasing and 30.8 million tonnes or 26% of the global fish supply was produced by aquaculture in 1998 (FAO, 2000). Global demand for food fish is increasing largely due to an escalating population in Asia, Africa and South America (Tidwell and Allan, 2001), however per capita consumption in developed countries is increasing also as the health benefits of eating fish are recognised (Treadwell et al., 1992).

There has been a long-term trend of increasing consumption of seafood in Australia from 4.9 kg/capita/year in the 1930s to 10.9 kg/capita/year in 1998/99 (ABS, 1998). However, Australia has very small capture fisheries by world standards and despite being a net value exporter of edible seafood (mostly high-value seafood such as rock lobsters, abalone and scallops), there is a deficit in supply of seafood for domestic markets (Allan, 1999). To offset this demand more than 108,639 tonnes of edible seafood was imported to Australia in 2000/01 (ABARE, 2002). In addition, aquaculture has experienced rapid growth in recent years (Allan, 1999). In 2000/01 the total aquaculture production of edible seafood in Australia was 43,602 tonnes, representing 19.0% of the total fisheries production (229,841 tonnes) and 23.2% of the total value (AUD$2.48 billion) (ABARE, 2002).

Production from capture fisheries in Australia is static and unlikely to increase in the future (McLoughlin et al., 1995). Therefore future increased demand for seafood in Australia, as a result of increased per capita consumption and/or predicted population

growth, and competition for imported seafood from other importing countries, will need to be met by aquaculture production (Allan, 1998).

Culture of Australian marine finfish occurs over a wide range in latitude, from coldwater southern Australia to tropical northern Australia. More than twenty marine fish species are cultured commercially or are under investigation for their aquaculture potential (Battaglene and Fielder, 1997), but production is dominated by three species. These are Atlantic salmon, *Salmo salar* in Tasmania (12,223 tonnes valued at AUD$95.3 million in 2000/01), Southern bluefin tuna, *Thunnus maccoyii* in South Australia (9,051 tonnes valued at AUD$263.8 million in 2000/01) and barramundi (=Asian sea bass), *Lates calcarifer* in Queensland, New South Wales and South Australia (913 tonnes valued at AUD$8.6 million in 2000/01) (ABARE, 2002). The seacage growout of salmon and tuna has developed rapidly since it began in the late 1980s largely due to the availability and translocation of proven technology from the northern hemisphere, availability of excellent seacage sites with high water quality, and good government support (Treadwell et al., 1991, Battaglene, 1995). Barramundi production started in 1984 using adaptations of intensive larval rearing technology developed in south-east Asia (McKinnon, 1987). Barramundi fingerlings are now cultured using a range of intensive, semi-intensive and extensive hatchery techniques and market-size fish are produced in marine seacages, freshwater ponds and intensive, recirculation facilities.

There is also significant interest to develop marine finfish aquaculture in New South Wales, which is the most populated state and has the largest domestic seafood market at Sydney (Quartararo, 1996; Allan, 1998). However, temperate coastal conditions in New South Wales are unsuitable for culture of salmonids and tropical species (Battaglene, 1995) and selection of other suitable fish species is necessary.

The sparid, Australian snapper, *Pagrus auratus* is an excellent candidate for large-scale, commercial aquaculture in temperate Australian conditions for several reasons (Battaglene and Bell, 1991). Firstly, snapper is a high profile recreational and commercial species in Australia and New Zealand and commands high market prices (Bell et al. 1991; Pankhurst et al., 1991; Francis 1994; Kable, 1996). There is a substantial short-fall in the supply of snapper to markets in Australia, and catches in the New South Wales fishery have declined from 1,000 tonnes in 1980 to 271 tonnes in 2000/01 (ABARE, 2002). This deficit is met by importation of more than 1,200 tonnes/year of snapper from New Zealand. The export of live fish from Australia is also developing and the value of the product sold increased from AUD$4.6m in 1992/93 to AUD$41.6m in 2000/01 (ABARE, 1995; 2002). A significant high value (AUD$14.50-17.50/kg) domestic market in the eastern states for live snapper also exists (Kable, 1996).

Secondly, the general biology of snapper, particularly in New Zealand is well known (e.g. Pankhurst and Carragher, 1992; Scott and Pankhurst, 1992; Scott et al., 1993; Francis, 1994; Pankhurst, 1994a; Battaglene, 1995; Willis et al., 2001) and research has been completed to develop techniques for snapper aquaculture. It is now possible to spawn high-quality eggs year-round from captive broodstock (Cleary, 1997; Fielder et al., 1999) and rear larvae to metamorphosis (Battaglene and Talbot, 1992; Battaglene and Allan, 1994). The growth of juvenile snapper in tanks and pilot-scale seacages has been assessed (Bell et al., 1991; Quartararo, 1996) and formulated diets have been developed (Quartararo et al., 1992; Allan and Quartararo, 1996).

Thirdly, snapper is closely related to the Japanese red sea bream, *Pagrus major*. Paulin (1990) suggested that snapper and red sea bream were the same species but were present in isolated southern and northern populations, respectively. However, more recently, Tabata and Taniguchi (2000) analysed samples of red sea bream from two Japanese areas, the East

China Sea and the South China Sea, and samples of snapper from Australia and New Zealand using restriction fragment length polymorphism analysis and DNA direct sequencing of the mtDNA control region. These authors suggested that the relationship between snapper and red sea bream is at the level of a subspecies and proposed accordingly that snapper be renamed *P. auratus auratus* and red sea bream be renamed *P. auratus major*.

Regardless of the taxonomy, a great deal is known about red sea bream biology, and technology is well developed for large-scale aquaculture production in Japan (see review by Foscarini, 1988). Approximately 96 million red sea bream fingerlings are produced each year in Japan for growout and stock enhancement (Fushimi, 2001). Translocation of the Japanese technology may assist with development of snapper aquaculture. In addition, technology developed for large-scale aquaculture of other sparids such as gilthead sea bream, *Sparus aurata* in the Mediterranean may also be suitable for snapper farming (Shields, 2001). However, the translocation of technology between countries is not always easy and can be inappropriate depending on species, cost of production and available infrastructure (Treadwell et al., 1992; Battaglene, 1995). Subtle differences in the optimal spawning temperature range for red sea bream (15-21°C) and snapper (19-23°C) also suggests that the environmental requirements for snapper larval rearing are different from that of red sea bream (Foscarini, 1988; Fielder et al., 1999).

After initial pilot-scale ventures in New South Wales and Western Australia in 1992 and 1994, respectively, commercial aquaculture of snapper has commenced in New South Wales, South Australia and Western Australia, and new ventures are planned for southern Queensland. However, production has been slow to take-off and two major bottlenecks threaten the viability and expansion of the snapper farming industry. The first is a lack of supply of cheap, healthy fingerlings and the second is a lack of access to suitable seacage

growout sites. Therefore, it is necessary to improve snapper larval rearing techniques and to identify new, alternative growout sites in Australia. There is also a need to develop cheaper, environmentally-friendly formulated feeds.

1.2. Larval rearing

Production of snapper fingerlings in Australia has been based on a combination of the principles of intensive larval rearing developed for red sea bream in Japan and gilthead sea bream in France (Foscarini, 1988; Battaglene and Talbot, 1992; Chatain, 1997). This consists of broodstock management and spawning in land-based tanks, incubation of fertilised eggs and then culture of larvae under relatively controlled salinity, temperature and light conditions at high densities (50-150 larvae l^{-1}) in 2,000-l to 10,000-l, clearwater tanks. High volumes of tank water are recirculated through mechanical and biological filters and particular attention is paid to minimising turbulence in the water column and removal of surface films. Larvae are fed nutritionally enriched live feeds; rotifers, *Brachionus rotundiformis* and *B. plicatilis* followed by *Artemia* metanauplii; for about 30 days after hatching and then weaned off live feeds onto commercial, formulated, pellet diets. Larvae usually remain in the hatchery tanks for 30-40 days after hatching, until metamorphosis has occurred and fish can be transferred to larger nursery tanks or cages for size-grading and ongrowing. Significant problems have occurred during larval rearing in all Australian hatcheries and only relatively small numbers of fingerlings (hundreds of thousands) have been produced to date (Battaglene, 1996).

In order to reduce the cost of fingerling production growth of larvae must be optimal to ensure that turn-over of larvae batches is maximised and more hatchery runs are completed each year. Survival of larvae must also be optimised and predictable. Survival of red sea bream and gilthead sea bream larvae from stocking to metamorphosis and weaning in

intensive systems is approximately 20-40% (Tandler, 1993; Chatain, 1997; Fushimi, 2001). Survival of snapper larvae, however, has been unpredictable and highly variable between batches, ranging from 0.8 to 68% (Battaglene, 1996). The optimal growth of snapper larvae in intensive tanks is not known.

Many factors can influence the success of intensive fingerling production and good knowledge of specific environmental and dietary requirements of larvae, and disease control is required if low volume, clear water, high exchange larval rearing methods are to be successful (Blaxter, 1988; Shepherd and Bromage, 1988; Barnabe, 1990; Battaglene, 1996; Chatain, 1997; Shields, 2001). It is likely that these requirements are species-specific and they may change during larval ontogeny (Barnabe, 1990; Shields, 2001). Several of these factors have already been investigated for snapper production.

The nutritional requirements, in particular n-3 HUFA levels, of Japanese red sea bream larvae are understood well (Kitajima et al., 1980; Watanabe et al., 1983,1989; Izquierdo et al., 1989; Watanabe 1993; Furuita et al., 1996). Techniques and commercial products for enrichment of live feeds, and weaning from live to artificial diets, are also well-developed (Sorgeloos and Leger, 1992) and Battaglene et al. (1993) showed that mortality of snapper larvae, particularly at weaning, was significantly reduced when live food enhancement techniques were used. The importance of low water turbulence and removal of surface films to optimise swimbladder inflation of snapper larvae has also been demonstrated (Foscarini, 1988; Battaglene and Talbot, 1992).

Determination of the optimal environmental conditions for larvae and juvenile fish is necessary to maximise production in hatcheries. Three of the most important physical parameters for growth and survival of fish are photoperiod, salinity and temperature (Barnabe, 1990; Battaglene, 1996; Hart et al., 1996; Howell et al., 1998; Boeuf and Le Bail, 1999). Shepherd and Bromage (1988) state that ideally all factors influencing larval

survival should be tested experimentally to find optimal rearing protocols; however, in reality, this level of sophistication is rarely accomplished. This research can be logistically difficult and expensive, as high replication of many treatments, often in factorial combinations, is required. Nevertheless, specific rearing protocols have been developed for several species including European sea bass, *Dicentrarchus labrax* and gilthead sea bream (Barnabe, 1990). This level of technical knowledge has enabled farming of these species to become the largest marine fish aquaculture industry in the Mediterranean and approximately 450 million fingerlings were produced in 1999 (Shields, 2001).

Adult snapper are marine fish and are generally found on inshore and offshore rocky reefs to depths <200 m (Paulin, 1990). In contrast, juvenile snapper occupy estuarine seagrass communities and coastal embayments (Battaglene and Bell, 1991). The environmental conditions in which Australian snapper larvae are typically reared, are based principally on ambient spring/summer coastal conditions when snapper are known to spawn (Battaglene and Talbot, 1992). Photoperiod, salinity and temperature during this time are approximately 14L:10D (hours light:dark), 35‰ and 16-23°C, respectively (Pankhurst et al., 1991; Battaglene, 1995). However, the photoperiod, salinity and temperature combination for optimal development, growth, and survival of snapper larvae in tanks is unknown.

1.2.1. Photoperiod and larval performance

Most marine fish larvae require light to feed (Pankhurst, 1994b; Pankhurst and Hilder, 1998; Cox and Pankhurst, 2000) and growth and survival can be affected by photoperiod (Boeuf and Le Bail, 1999). Provision of light phases longer than ambient conditions has improved growth of several cultured marine fish larvae, presumably by extending the period of active visual feeding and increasing the number of ingestion/digestion events

through the day (Duray and Kohno, 1988; Barahona-Fernandes, 1979; Ronzani Cerqueira and Chatain, 1991; Barlow et al., 1995; Hart et al., 1996). Initiation of growth at the start of exogenous feeding is also affected by the ability of marine fish larvae to catch prey. First-feeding can occur on the first day for some species of larvae, or in some cases such as gilthead sea bream larvae, may take up to 2-3 d to learn (Tucker, 1988; Parra and Yúfera, 2000). Longer photoperiods therefore provide more opportunity for larvae to develop feeding skills. However, optimal photoperiods are species-specific and maximal hours of feeding is not necessarily the most important factor of optimal conditions (Blaxter, 1986). Factors such as excessive ingestion of live feeds and consequent reduced assimilation in extended photoperiods (Howell et al., 1998), as well as potential circadian feeding rhythms and periods of larval inactivity regardless of the presence of light, may also influence the effects of photoperiod on fish larval growth (Ronzani Cerquiera and Chatain, 1991).

Similarly, optimal photoperiod for survival of larvae is species-specific and survival can be reduced in extended photoperiods (Barahona-Fernandes, 1979; Ronzani Cerqueira and Chatain, 1991). The success of some fish larvae to inflate their swimbladders has been correlated negatively with exposure to light (Battaglene and Talbot, 1990). Swimbladder inflation of snapper larvae and many other physoclistous marine fish larvae starts when endogenous yolk-sac reserves are depleted and exogenous feeding begins and may continue for several days until the pneumatic duct closes (Hunter and Sanchez, 1976; Kitajima, et al., 1981; Chatain and Ounais-Guschemann, 1990; Battaglene, 1995). Larvae must swim to the water surface and gulp air, therefore light can discourage negatively phototactic larvae from swimming to the surface (Ronzani Cerqueira and Chatain, 1991; Battaglene, 1995). Larvae without functional swimbladders can fail to thrive, display high degrees of spinal deformities and experience significant stress-related mortality (Chapman et al., 1988; Chatain and Dewavrin, 1989; Battaglene and Talbot, 1990, 1992; Chatain,

1994; Kitajima et al., 1994; Trotter et al., 2001). Therefore, conditions for optimal swimbladder inflation are a compromise between periods of darkness when larvae can swim to the surface and periods of light when larvae can actively feed. Once the milestone of swimbladder inflation has been achieved, the optimal photoperiod for performance of larvae reared in tanks may change.

1.2.2. Salinity and temperature and larval performance

Water salinity and temperature can affect directly the survival and normal development of fish and the biological effects of salinity and temperature are correlated in various ways (Alderdice, 1988; Blaxter, 1988). For example, salinity can modify the effects of temperature and widen, narrow or shift the tolerable temperature range; temperature can modify the effects of salinity accordingly (Kinne, 1963). Therefore, due to the interactive effects of salinity and temperature on osmoregulation they should be considered together when determining optimal conditions for tolerance and performance of fish (Kinne, 1963). Although many marine fish are euryhaline and eurythermic the various development states of fish (fertilised egg, larva and juvenile) may have specific and/or different salinity and temperature optima and tolerance may be influenced by maternal conditions (Blaxter, 1969; Alderdice, 1988; Blaxter, 1988; Battaglene, 1995; Howell et al., 1998). Tolerance to rapid changes in salinity and temperature may also be influenced by the state of fish development.

In general, marine teleost fish are hypoosmotic regulators and maintain osmolality of their body fluids lower than the external environment (Alderdice, 1988). Regulation of excess Na^+ and Cl^-, which are ingested as marine fish drink seawater to replace osmotic water loss, is an active process requiring energy and occurs through specialised chloride cells (Alderdice, 1988). Chloride cells are mostly located in the branchial epithelium,

however in larvae with undeveloped gills, including red sea bream, these cells occur in integumental tissues (Yamashita, 1978; Hwang and Hirano, 1985). In general, chloride cells proliferate in seawater and degenerate in freshwater (Laurent and Dunel, 1980). Larvae, when challenged with varying environmental salinity, must alter their osmoregulatory mechanisms to maintain homeostasis. Survival of larvae has been correlated with the ability for larvae to modify morphology of their chloride cells and this varies with species and the degree of change in environmental salinity (Hwang and Hirano, 1985).

Salinity can affect larval utilisation of yolk, growth rate and survival by influencing the amount of energy needed for osmoregulation (Howell et al., 1998). Metabolic demand to maintain homeostasis increases as environmental salinity is increased, therefore in some euryhaline species larvae are bigger at intermediate, near-isoosmotic salinities (Howell et al., 1998).

Salinity can also affect buoyancy of eggs and larvae which in turn can influence the ability of larvae to swim to the water surface to gulp air and inflate their swimbladders (Hadley et al., 1987; Battaglene and Talbot, 1990, 1993). Low salinity can reduce larval buoyancy and inhibit their ability to swim to the water surface (Battaglene and Talbot, 1990), whereas osmotic stress associated with high salinity (~40-45‰) can also reduce swimbladder inflation in gilthead sea bream and European sea bass (Tandler et al., 1995; Johnson and Katavic, 1984).

Calculi in the urinary bladder of marine fish larvae have been observed in a number of species including the sparids; snapper, sharpsnout seabream, *Diplodus puntazzo* and gilthead sea bream (Modica et al., 1993; Battaglene, 1995; Favaloro and Mazzola, 2000). Although urinary calculi have been rarely associated with larval mortality and reduced

growth (Modica et al., 1993), their formation suggests inappropriate environmental conditions or a degree of physiological dysfunction.

Temperature usually has a greater effect on fish performance than salinity. In general, temperature changes have a more profound effect during early life, and embryos and larvae tend to be more stenothermal than juveniles and adults (Rombough, 1988; Rombough, 1996). Temperature can affect virtually every aspect of fish reproduction (van Der Kraak and Pankhurst, 1996) and larval development including hatching size, efficiency of yolk utilisation, growth, feeding rate, gut evacuation time and digestion rates, time to metamorphosis, metabolic demand, oxygen consumption, behaviour, and swimming speed (Blaxter, 1988; Ronzani Cerqueira, 1991; Rombough, 1996). Within the tolerable range, larval growth tends to increase as temperature is increased (Person Le-Ruyet and Verillaud, 1980; Tandler et al., 1989; Hart et al., 1996). However, larvae reared near the extremes of their tolerable range or indeed only a few degrees Celsius higher or lower than the optimal temperature can experience major developmental abnormalities such as jaw and operculum deformities (Pittman et al., 1990; Battaglene, University of Tasmania, pers. comm., 2002).

Little is known of the effects of varying temperature on swimbladder inflation on marine fish larvae (Battaglene, 1995), but the optimal temperature window for swimbladder inflation can be very narrow (Trotter et al., in press). In addition, several studies have shown that organogenesis, including swimbladder inflation, varied with temperature and was slightly better at lower temperatures within the tolerable range (Gibson and Johnston, 1995; Hadley et al., 1987).

1.2.3. Commercial-scale evaluation

Many studies have investigated the short-term effects of photoperiod, salinity and temperature on marine fish larvae (for reviews see: Alderdice, 1988; Blaxter, 1988; Rombough, 1996; Boeuf and Le Bail, 1999). Most of these studies have been done in small tanks ranging in size from 1-l to 150-l (Perschbacher et al., 1990; Parado-Estepa, 1991; Hart et al., 1996; Bolla and Ottesen, 1998; Specker et al., 1999; Steinarsson and Björnsson, 1999) probably for logistical reasons to allow replication of several treatments. Larval stocking densities are also generally low to minimise the potential influence of density-related factors such as feed availability and cannibalism on larval growth and survival (Battaglene, 1995). Few studies have determined the long-term effects of photoperiod, salinity and temperature on larval performance (Tandler et al., 1995; Hart et al., 1996) and even fewer studies have been done in large, commercial-scale tanks (e.g. >600-l) at high (commercial) stocking densities.

The final large-scale, commercial evaluation/validation of results from rigorous small-scale experiments is important to obtain information on the potential number and costs of juvenile fish production in hatcheries as well as identifying the need for novel infrastructure and determining the logistics of scaling-up from experimental to commercial hatchery production. Other factors such as light intensity, turbulence and aeration which also influence performance of fish larvae (Chatain and Ounais-Guschemann, 1991; Opstad and Bergh, 1993; Huse, 1994; Battaglene, 1995; Cobcroft et al., 2001) may differ with tank size. The influence of small tank environmental parameters in combination with the photoperiod, salinity and temperature effects may therefore affect fish larval performance to a greater or lesser degree in large-scale, commercial production tanks.

1.3. Alternative growout sites

Commercial floating sea-cage marine fish farms are operating in inshore and offshore sites in New South Wales, South Australia, Tasmania and Queensland. However, expansion of a sea-cage-based industry in Australia, and especially snapper farming in New South Wales, may be limited by a lack of sites with suitable water quality, water depth and proximity to land-based infrastructure (Ogburn, 1996; Brown et al., 1997; Allan, 1998). Much of the New South Wales coastline is heavily populated and many coastal areas suitable for aquaculture are used for recreational watersports or are set aside as national parks or marine reserves (Allan, 1998). The public community also has perceived concerns about environmental impact of sea-cage aquaculture and this coupled with conflict with other waterway users has already hindered approval of sea-cage farms by appropriate government authorities.

An alternative to seacage farming is development of land-based aquaculture in ponds or tanks. Farming of marine prawns in coastal ponds is well developed in northern NSW and southern Queensland (Allan, 1999) and it may be possible for some farmers to diversify into snapper farming. However, large-scale development of new, purpose-built coastal marine fish ponds is unlikely for reasons similar to those that will restrict sea-cage development (Allan, 1998).

Alternative, additional sites for farming marine species may be available if the large reserves of saline groundwater occurring in inland areas of Australia can be utilised. Approximately 2.5 million ha of land is affected by increasing saline groundwater discharge in Australia. Clearing of native trees and grasses and replacement with annual crops and pastures, irrigation schemes and poor water management in both rural and urban areas have meant that excess groundwater is mobilising deep soil salts and is bringing them

to the land surface and into rivers (Blackwell, 1999; Willett, 1999). Crop production is affected adversely and natural vegetation is destroyed.

The only current engineering solution to rising saline groundwater is the construction and operation of large-scale sub-surface drainage systems, which collect saline groundwater and dispose of it in a series of ponds ranging in size from 2-30 ha (Ingram et al., 1996; Allan and Fielder, 1999). The concentrations of salt of this water increases progressively as it flows from one pond to the next and eventually crystalline salt is deposited.

In Australia, eleven saline groundwater sub-surface drainage schemes, which incorporate more than 6,000 ha of evaporation ponds are operating in the Murray-Darling Basin and a further eight schemes are planned for construction. The largest of these schemes is the Wakool-Tullakool Sub-Surface Drainage Scheme (WTSSDS) where approximately 13,000 Ml yr^{-1} of saline groundwater is pumped into 1,600 ha of evaporation ponds (Ruello, 1996). In addition, 76 towns in Australia were identified as being threatened by salinity, including 21 towns in New South Wales, and construction of saline groundwater schemes with evaporation ponds is being considered in many of these towns (Allan et al., 2001).

Saline groundwater sub-surface drainage schemes are expensive to build and operate. However, if the saline groundwater and environmental conditions are suitable, a unique opportunity to utilise the water for culture of aquatic organisms and to develop a new aquaculture industry is presented. Aquaculture development could provide significant opportunities for new economic development and employment in rural areas and also help to offset some of the costs involved with constructing and operating the saline groundwater mitigation schemes.

Saline groundwater has been used successfully in the United States and the Middle East to culture a range of algae, crustaceans and finfish such as tilapia, *Oreochromis* spp., red drum, *Sciaenops ocellatus*, sea bream, eels, *Anguilla* spp. and channel catfish, *Ictalurus punctatus* (Forsberg et al., 1996; Ingram et al., 1996; Samocha et al., 1998). In Arizona, USA, a new prawn, *Penaeus vannamei* farming industry is emerging that is totally dependent on saline groundwater. Salinity of the groundwater ranges from 2-6‰, between 80 and 100 ha of ponds have been constructed and over 120 tonnes of prawns were produced in 2001 (Allan and Fielder, 2002).

In Australia, saline groundwater from shallow and deep aquifers has been suitable for short-term growth and survival of diadromous finfish such as Australian bass, *Macquaria novemaculeata*, barramundi and Atlantic salmon (Allan and Fielder, 1999; Gooley et al., 1999). To date, attempts have not been made to culture marine finfish like snapper in Australian saline groundwater.

The quality of saline groundwater in shallow aquifers can vary significantly within relatively short distances of catchment areas and may range in salinity from < 1‰ to 35‰ (Allan and Fielder, 1999; Gooley et al., 1999; Nulsen, 1999). The concentration of ions and their ratios in saline groundwater can also vary with location (Forsberg et al., 1996; Nulsen, 1999). Australian saline groundwater is dominated by Cl^- and Na^+, which are present in concentrations similar to that of equivalent salinity seawater, however the concentrations of other major ions, such as Mg^{2+}, Ca^{2+} and K^+, can vary widely from that of seawater (Allan and Fielder, 1999; Nulsen, 1999). In saline groundwater from the WTSSDS, the $[K^+]$ is approximately 4.5% of that found in equivalent strength seawater (Allan and Fielder, 1999).

Potassium is the main cation of intracellular fluids while Na^+ and Cl^- are the main extracellular ions (Teeter, 1997). Successful osmoregulation, as characterised by constant

extracellular and intracellular osmolality, is largely determined by homeostasis of these ions. Seawater fish obtain most of their K^+ requirement through ingestion of seawater and food (Sakamoto and Yone, 1978; Wilson and El Naggar, 1992; Lall, 1989) but K^+ can also be sequestered from the environment by diffusion across the branchial epithelium (Sanders and Kirschner, 1983; Gardaire et al., 1991). The performance of snapper in low potassium saline groundwater from the WTSSDS was not known before the current investigation.

1.4. Osmoregulation

The range in salinity in coastal seawater or inland saline groundwater aquaculture ponds can vary widely depending on initial water quality, rainfall and evaporation. In addition, the ionic composition of inland saline groundwater can vary. Euryhalinity or the ability to maintain homeostasis in a wide range of salinities is an attribute which has enhanced the suitability of several fish species, including sparids, for aquaculture (Wu and Woo, 1983). Knowledge of the ability of fish to osmoregulate in different environmental osmolalities and water of differing chemistry is therefore necessary to allow selection of suitable sites and pond management protocols. The tolerance and osmoregulatory ability of snapper to salinity change and low K^+ inland saline groundwater was not known before the current investigation.

Marine fish in seawater are hypoosmotic to the surrounding environment and tend to lose water by osmosis and gain Na^+ and Cl^- by diffusion. To compensate for the water loss, seawater-adapted teleosts drink seawater and then maintain ionic homeostasis of extracellular fluid by eliminating excess ingested ions. Most divalent ions remain in the gut and are passed with faeces while excess monovalent ions (Cl^-, Na^+) are absorbed into the blood and actively secreted from the chloride cells of branchial surfaces (Alderdice, 1988; McCormick, 1995).

Fish challenged with an altered environmental osmolality must maintain their body osmolality and ionic balance by changing behaviour, such as drinking rate (Tytler and Blaxter, 1988; Ura et al., 1996; Miyazaki et al., 1998) and functions of the osmoregulatory surfaces (Hwang and Hirano, 1985; Hwang et al., 1989; Arai et al., 1997; Perry, 1998; Kelly and Woo, 1999).

Chloride cells or "mitochondria-rich cells" are highly specialised cells that perform a range of functions to maintain ionic and acid-base balance in fish (Fig. 1.1). In seawater-adapted fish chloride cells are characterised by an extensive tubular system which is continuous with the basolateral membrane. Na^+,K^+-ATPase, located in high concentrations on the basolateral membrane and tubular system, actively pumps Na^+ out of a chloride cell into the tubular system lumen and consequently creates a highly negative intracellular charge (Fig. 1.1 [1]). Na^+ which enters the cell is recycled for K^+ via the Na^+,K^+-ATPase (Fig. 1.1 [1]). It is likely that recycling of K^+ across the basolateral side of chloride cells occurs through K^+ channels but the pathway is not described (Fig. 1.1 [2]). The Na^+ gradient is then used to transport Cl^- into the cell from the blood through a secondary $Na^+/K^+/2Cl^-$ cotransporter, which is also located in the tubular membrane (Fig. 1.1 [3]). The Cl^- leaves the cell on an electrical gradient through an apical Cl^- channel (Fig. 1.1 [6]). Na^+ is transported through a paracellular pathway down its concentration gradient (plasma being more positive than seawater) (Fig. 1.1 [PC]). Excretion of NH_4^+ may be achieved by NH_4^+ binding to the K^+ extracellular site of Na^+,K^+-ATPase and the K^+ binding site of the $Na^+/K^+/Cl^-$ cotransporter and being actively and passively transported into the cellular compartment, respectively (Fig. 1.1 [1] and [2]). Calcium most likely passively enters the chloride cell across the apical membrane through non-voltage-dependent Ca^{2+} channels (Fig.1.1 [9]) but this remains to be confirmed. In contrast, translocation of Ca^{2+} across the basolateral membrane is actively transported by Ca^{2+}-ATPase (Fig. 1.1 [8]) as well as by

Na^+/Ca^{2+} exchange which is driven by the transmembrane Na^+ gradient (Fig. 1.1 [7]). Control of pH in chloride cells is most likely controlled by basolateral Na^+/H^+ and Cl^-/HCO_3^- exchanges (Fig. 1.1 [11]) (see reviews by Pisam and Rambourg, 1991; McCormick, 1995).

The number, size and location of branchial chloride cells are known to alter with changes in environmental salinity, however the degree of change in cells can be influenced by the range of change in salinity as well as the species and age of fish (Hwang and Hirano, 1985; Kelly and Woo, 1999; Kelly et al., 1999; Caberoy and Quinitio, 2000). Transfer of euryhaline fish from freshwater to saltwater generally causes a proliferation of branchial chloride cells and an increase in branchial Na^+, K^+-ATPase activity (Sasai et al., 1998). Two distinct types of chloride cells have been observed in gill filament and lamellar epithelia of several species of diadromous and euryhaline teleosts (Uchida and Kaneko, 1996; Uchida et al., 1996; Uchida et al., 1997; Sasai et al., 1998; Hirai et al., 1999). Changes in morphology of chloride cells following transfer to saltwater and freshwater suggest that filament and lamellar chloride cells are important in saltwater and freshwater osmoregulation, respectively. Both lamellar and to a lesser degree filament chloride cells of several species of fish have also proliferated following exposure to ion-poor freshwater (Laurent and Dunel, 1980; Perry and Laurent, 1989; Greco et al., 1995; Perry, 1997).

Several studies have investigated the effects of low environmental $[K^+]$ in freshwater (Gardaire et al., 1991) and artificial seawater (Sanders and Kirschner, 1983; Marshall and Bryson, 1998) on freshwater- and seawater-adapted fish, or excised epithelial tissue, to provide information on the kinetics and transport mechanisms of K^+ in chloride cells. However, no reports of studies to determine the effects of low environmental K^+ in a saline environment on osmoregulation and chloride cell morphology of a euryhaline fish were found.

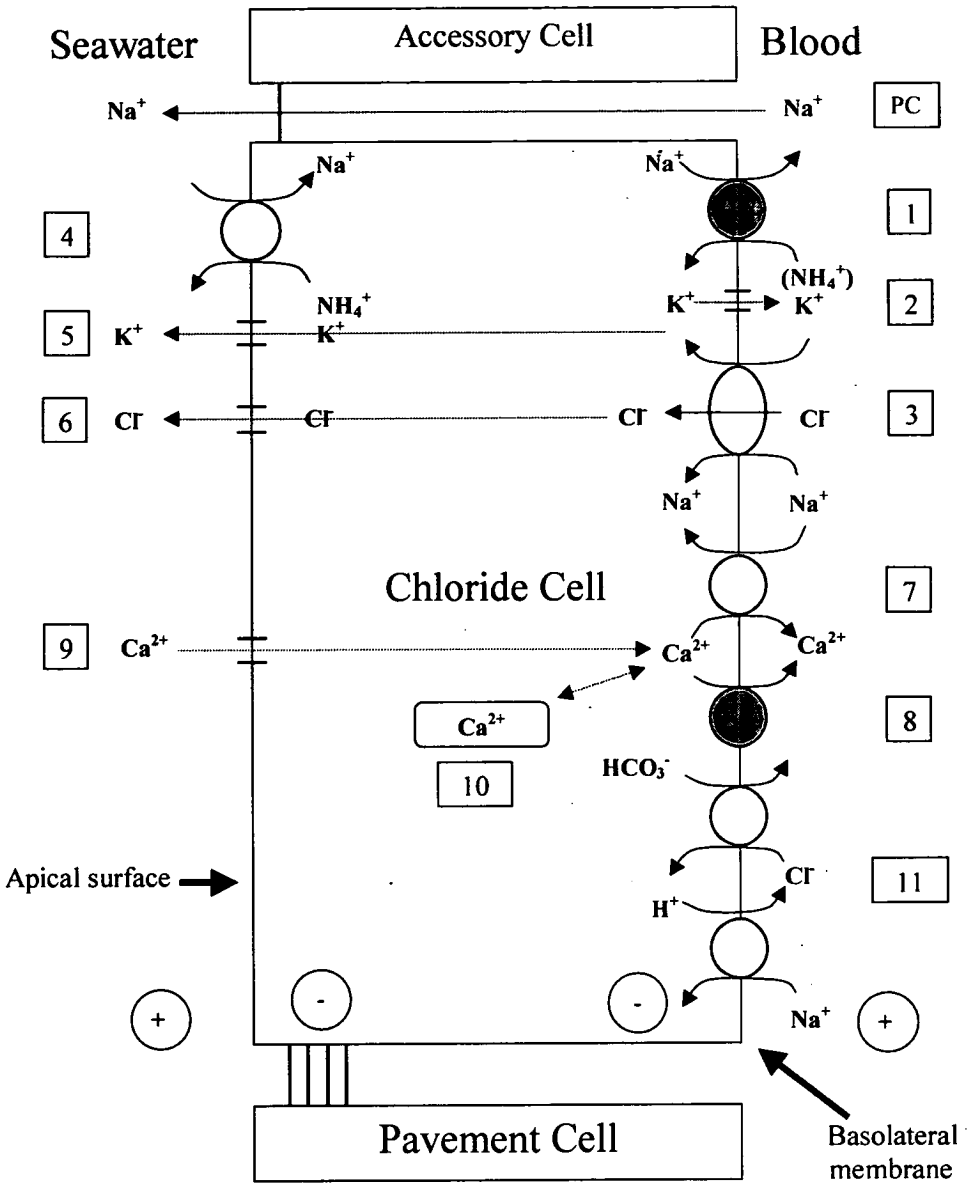

Fig. 1.1. Model of seawater chloride cell functions, re-drawn and modified from Marshall and Bryson (1998). Shaded circles indicate active ion transport ATPases; open shapes indicate passive transport processes driven by transmembrane chemical gradients. (PC) paracellular pathway, (1) Na^+,K^+-ATPase, (2) basolateral K^+ channels, (3) $Na^+/K^+/2Cl^-$ cotransporter, (4) Na^+/NH_4^+ exchange may mediate NH_4^+ excretion, (5) a small number of apical K^+ channels accounts for K^+ secretion, (6) apical anion channels for Cl^- secretion, (7) Na^+/Ca^{2+} exchange mediates part of Ca^{2+} uptake, (8) Ca^{2+}-ATPase accounts for the balance of Ca^{2+} uptake, (9) apical Ca^{2+} channels, (10) intracellular Ca^{2+} can be mobilised from intracellular pools, (11) intracellular pH is regulated by Cl^-/HCO_3^- exchange and Na^+/H^+ exchange.

1.5. Scope and aims of this thesis

The overall aim of research described in this thesis was to develop techniques to improve the large-scale production of juvenile snapper and evaluate the suitability of inland saline groundwater for land-based growout of snapper in New South Wales.

This was done by assessing:

(a) The effects of photoperiod, salinity and temperature on larval development, growth and survival in small-scale experiment tanks.

(b) The combined effects of optimal photoperiod, salinity and temperature identified in experiment tanks on larval performance in commercial-scale tanks.

(c) The growth and survival of juvenile snapper in inland saline groundwater from New South Wales.

(d) The effect of salinity on osmoregulation of juvenile snapper.

(e) The effect of potassium deficient inland saline groundwater on osmoregulation of juvenile snapper.

The objectives were to identify the optimal regime for intensive larval rearing to maximise hatchery production of juvenile snapper, to identify the aquaculture potential for snapper in estuarine and inland saline groundwater ponds, to increase the understanding of osmoregulation and mechanisms for adaptation of snapper to different environmental salinities, and to contribute to the understanding of osmoregulation of marine teleosts in ion-deficient saline environments

The thesis is presented as a series of papers in the form that they have been submitted for publication and are referred to in the text as Chapters 2 to 7. The chapters are either submitted for publication or have been published* as described below. Chapter 1 is a

general introduction and provides information on the current status and constraints for development of fish farming in Australia. This chapter also outlines the aims and objectives of the thesis. Chapter 8 is a general discussion and results are discussed in relation to the larval rearing and osmoregulation of other sparids and the potential for aquaculture of snapper in inland saline groundwater. Recommendations for future research are also made. References for Chapters 1 and 8 are furnished at the end of the thesis.

***Chapter 2:** Fielder, D.S., Bardsley, W.J., Allan, G.L., Pankhurst, P.M., (2002). Effect of photoperiod on growth and survival of snapper *Pagrus auratus* larvae. Aquaculture 211, 135-150.

Chapter 3: Fielder, D.S., Bardsley, W.J., Allan, G.L., Pankhurst, P.M., (Submitted). The effects of salinity and temperature on growth and survival of Australian snapper, *Pagrus auratus* larvae. Aquaculture.

Chapter 4: Fielder, D.S., Allan, G.L., Pankhurst, P.M., (Submitted). The combined effects of salinity, temperature and photoperiod on growth and survival of Australian snapper, *Pagrus auratus* larvae in commercial-scale tanks. J. World Aquacult. Soc.

***Chapter 5:** Fielder, D.S., Bardsley, W.J., Allan, G.L., 2001. Survival and growth of Australian snapper, *Pagrus auratus*, in saline groundwater from inland New South Wales, Australia. Aquaculture 201, 73-90.

Chapter 6: Fielder, D.S., Allan, G.L., Pepperall, D., Pankhurst, P.M., (Submitted). The effects of changes in salinity on osmoregulation and chloride cell morphology of juvenile Australian snapper, *Pagrus auratus*. Aquaculture.

Chapter 7: Fielder, D.S., Allan, G.L., Pepperall, D., Pankhurst, P.M., (Submitted). The effects of potassium concentration in saline groundwater on osmoregulation

and chloride cell morphology of juvenile Australian snapper, *Pagrus auratus*. Aquaculture.

As leader of a NSW Fisheries research and development program for marine fish breeding, I was responsible for operation of all aspects of broodstock management, spawning, larval rearing, live feed production and maintenance of juvenile fish. I supervised daily operations of these production areas and contributed on a daily basis to the general hands-on aspects of the operations. I received other technical assistance from technicians employed at NSW Fisheries Port Stephens Fisheries Centre with these duties.

All experiments described in this thesis were designed and operated on a daily basis by me. I sourced all equipment and chemicals necessary for the experiments and established all of the necessary tanks and laboratory space. I did all aspects of data collection for experiments including microscopic examination of larvae, measurement and weighing of larvae (Chapters 2, 3 and 4), water collection and chemical manipulation of groundwater, stocking of experiments (Chapter 5), processing of blood to provide serum, dissection, and fixing of gill filaments, photography and image analysis of gill filaments (Chapters 6 and 7) and collation and statistical analyses of all experimental data.

I received hands-on assistance from technicians with duties including harvest of larvae and stocking of experiment tanks, collection of water quality data, provision of live feeds (rotifers) for larval feeding, harvest of experiment tanks and counting of larvae ad juvenile fish, recording (scribing) of data, extraction of blood from fish in experiments described in Chapters 6 and 7.

I contracted a NATA approved laboratory, Hunter Water Laboratories, to do analyses of seawater and saline groundwater ion concentrations described in Chapter 5. Ms Debbie Pepperall of Newcastle University, Department of Anatomical Pathology provided her technical expertise to section gill filaments and immunocytochemical staining of the gill

sections. I assisted Ms Pepperall in the preparation of gill samples. The serum osmolality and electrolyte analyses were done by a NATA endorsed laboratory, Hunter Area Pathology Service.

Ethical clearance to do this research was provided under permits for Project Numbers 93/1, 93/3, 94/6, 97/6 and 99/16 of the NSW Fisheries Animal Care and Ethics Committee.

CHAPTER 2

Effect of photoperiod on growth and survival of snapper *Pagrus auratus* larvae

2. Effect of photoperiod on growth and survival of snapper *Pagrus auratus* larvae

2.1. Summary

Experiments were done in 100-l recirculation tanks to determine the effects of photoperiod on (1) first-feeding and (2) post-swimbladder inflated snapper, *Pagrus auratus* larvae. In Experiment 1, feeding onset, growth, initial swimbladder inflation and tail flexion were assessed at five photoperiod treatments (0L:24D, 6L:18D, 12L:12D, 18L:6D and 24L:0D) in larvae from 3-15 days after hatching (dah). Growth and development of first-feeding larvae increased with increasing photoperiod duration in the 12L:12D to 24L:0D treatments. Larvae did not start feeding in 0L:24D and onset of feeding was delayed by up to 3 d in 6L:18D. All larvae held in 0L:24D and 6L:18D died within 6 or 9 dah, respectively. Initial swimbladder inflation was best (80-100%) in an intermediate photoperiod of 12L:12D at 9 dah. By 15 dah, although the percentage of larvae with inflated swimbladders had increased in all treatments, swimbladder inflation in 12L:12D was 1.3 and 2.0 times greater than that of larvae in 18L:6D and 24L:0D, respectively. In the second experiment, growth and survival of snapper after the initial swimbladder inflation period (11-32 dah) were assessed at three photoperiod treatments (12L:12D, 18L:6D and 24L:0D). Growth was greatest in 18L:6D in which wet weights (16.3 ± 0.5 mg; mean ± S.E.) and dry weights (2.8 ± 0.1 mg; mean ± S.E.) of larvae were approximately 1.3 and 1.9 times heavier than larvae held in 24L:0D and 12L:12D, respectively. Survival of snapper larvae to 32 dah was not significantly different between the three photoperiod treatments; however, power of the experiment to detect effects on survival was low due to high variability within treatments. Further research is needed to determine optimal photoperiods for survival of snapper larvae. Because of the potential for high larval mortality if initial swimbladder inflation is not achieved, the optimal

photoperiod for the period from feeding onset to swimbladder inflation (3-15 dah) was deemed to be 12L:12D, whereas on the basis of growth parameters measured (total length, wet and dry weights), 18L:6D was determined to be the optimal photoperiod for culture of snapper from the post-swimbladder window to metamorphosis (11-32 dah).

2.2. Introduction

Determination of the optimal environmental conditions for larvae and juvenile fish is necessary to maximise production in hatcheries (Hart et al., 1996). One of the most important physical parameters for growth and survival of fish larvae is photoperiod (Barnabe, 1990; Chatain and Ounais-Guschemann, 1991; Battaglene, 1995; Hart et al., 1996; Boeuf and Le Bail, 1999). Many marine fish larvae are visual predators and therefore require light for efficient planktivory (Blaxter, 1980; Boeuf and Le Bail, 1999). However, optimal photoperiod for larval development, and growth and survival may differ, and also change with larval ontogeny.

In general, long photoperiod improves performance of fish larvae, probably because of increased food availability (Boeuf and Le Bail, 1999). For example, photoperiods longer than that of ambient conditions increased growth of larval rabbitfish, *Siganus guttatus* (Duray and Kohno, 1988), sea bass, *Dicentrarchus labrax* (Barahona-Fernandes, 1979; Ronzani Cerqueira and Chatain, 1991), barramundi, *Lates calcarifer* (Barlow et al., 1995) and greenback flounder, *Rhombosolea tapirina* (Hart et al., 1996). Conversely, survival of larvae can be reduced in extended photoperiods (Barahona-Fernandes, 1979; Ronzani Cerqueira and Chatain, 1991). Decreased survival and increased prevalence of deformities of fish larvae can be associated with failure of larvae to inflate their swimbladders (Chatain and Dewavrin, 1989; Battaglene, 1995). Light intensity and photoperiod can both influence the ability for larvae to inflate their swimbladders, although this varies with species (Battaglene and Talbot, 1990; Ronzani Cerqueira and Chatain, 1991; Chatain and Ounais-Guschemann, 1990; Battaglene, 1995).

Snapper, *Pagrus auratus*, is an important commercial and recreational species found in Australian and New Zealand waters (Bell et al., 1991; Battaglene and Bell, 1991; Pankhurst et al., 1991). However, wild catches are declining in Australia (ABARE, 1998)

but aquaculture of snapper is increasing in Australia using intensive larval rearing techniques followed by growout in sea-cages (Battaglene and Talbot, 1992; Battaglene and Fielder, 1997). These techniques are similar to those used for culture of the closely related Japanese red sea bream, *P. major* (Foscarini, 1988; Tabata and Taniguchi, 2000).

The environmental conditions in which Australian snapper larvae are typically reared, are principally based on ambient conditions during the natural spring spawning season from August to November (Battaglene and Talbot, 1992). Photoperiod during this time ranges from approximately 10-14 h. However, the photoperiod for optimal development, growth, and survival of snapper larvae is unknown.

The aim of this study was to investigate the effects of photoperiod on larval snapper ontogeny to determine the optimal photoperiod protocol for swimbladder inflation, growth and survival of snapper larvae reared in tanks.

2.3. Materials and methods

Two laboratory experiments were done at the NSW Fisheries, Port Stephens Fisheries Centre from June to July 1999.

2.3.1. Source of larvae

Fertilised snapper eggs were obtained from first generation hatchery-reared (G1) broodstock held in a 17,000-l flat-bottomed tank, with an independent, recirculating system comprising an external 700-l egg collection sump, pump and mechanical and biological filters. The tank was housed in a temperature and photoperiod controlled room.

Two groups of snapper larvae (group 1 and 2) were produced on independent occasions after each of a 2.3 kg and a 2.2 kg female snapper was induced to spawn using intramuscular pellet implants containing 207 and 120 μg/kg LHRHa (des-gly^{10}, D-trp^{6},

pro^{9}-ethylamide; Peptech Animal Health Pty Limited, Australia), respectively. Fertilised eggs were collected from the collection sump and transferred to 2,000-l conical-bottomed tanks with black sides and a white bottom filled with sterilised seawater (20 mg l^{-1} sodium hypochlorite for 24 h then neutralised with sodium thiosulphate) for incubation under darkness, recirculation was at 5 l min^{-1}, pH, 7.5; dissolved oxygen (DO_2), 7.5 mg l^{-1}; temperature, 19.3-20°C; and salinity, 35.3‰.

Hatching occurred approximately 30 h post-fertilisation in both groups of larvae. Yolk-sac larvae from group 1 were held in the incubation tank until 2 days after hatching (dah) under a 14L:10D (L:D, h of light:dark) photoperiod provided by a fluorescent light (Philips White TLA 40W 33QS) at temperature range of 20.4-21.0°C and slight aeration (200 ml min^{-1}).

Larvae from group 2 were held in the 2,000-l incubation tank until 11 dah at a photoperiod of 12L:12D . Rotifers, *Brachionus plicatilis* enriched with the micro-algae *Pavlova lutheri* and Tahitian *Isochrysis* aff. *galbana*, and DHA Super Selco (Inve Aquaculture NV, Belgium) were fed to the larvae twice daily from the day of feeding onset (4 dah) at a density of 10 ml^{-1}. A surface skimmer was installed 5 dah to remove surface films and thus facilitate swimbladder inflation (Chatain and Ounais-Guschemann, 1990; Battaglene and Talbot, 1994). From 8–11 dah, approximately 25% of the tank was drained daily and refilled with fresh groundwater (0.6‰) to gradually reduce the salinity from 35‰ to 20‰. Previous research demonstrated that swimbladder inflation and survival of snapper larvae were not affected by a reduction in salinity but growth was increased when salinity was reduced from 35‰ to 20‰ (Chapter 3).

2.3.2. Tanks used for the photoperiod experiments

Experiments were done in 100-l tanks described by Fielder and Bardsley (1999). Surface skimmers were provided to each tank to remove surface films. Incandescent lighting (Osram Halogen Decostar 51 12V 50W) was provided overhead. Each tank was enclosed within a box made from black plastic sheet to prevent escape of light to surrounding tanks. Approximately 5% of the water in each tank was exchanged each day and salinity was maintained by adding rain water when needed.

2.3.3. Experiment 1: Effect of photoperiod from 3-15 dah

The aim of the experiment was to determine the effect of photoperiod on growth and development of first-feeding snapper larvae from 3 to 15 dah. The photoperiod treatments were: 0L:24D, 6L:18D, 12L:12D, 18L:6D and 24L:0D.

Larvae from group 1 (3 dah; 2.56 ± 0.23 mm TL, n = 10) were harvested from the 2,000-l tank then transferred to a 50-l tank in which they were mixed homogeneously by slowly raising and lowering a perforated 27-cm diameter plastic disc. Randomly selected 200-ml samples of larvae were then transferred into each of 30 100-l tanks. There were 6 randomly selected replicate tanks per photoperiod treatment. Physical tank parameters were maintained: pH range, 7.6-7.8; DO_2 range, 7.4-7.6 mg l^{-1}; temperature range, 20.4-21.0°C; salinity range, 34.7-35.3‰; light intensity, 13.6 ± 3.1 µmol s^{-1} m^{-2} (mean ± S.D., n = 30). Five additional 200 ml samples of larvae were taken to estimate initial stocking densities (1137 ± 80 larvae $tank^{-1}$; mean ± S.D.).

After stocking and in accordance with the reduction of salinity previously described for group 2 larvae, salinity was progressively reduced from 35.1 ± 0.3‰ (n = 30) to approximately 20‰ over 5 d by adding rainwater.

2.3.4. Experiment 2: Effect of photoperiod from post swimbladder inflation

The aim of the experiment was to determine the effect of photoperiod on growth and development of post-swimbladder inflated snapper larvae from 11 to 32 dah. The photoperiod treatments were: 12L:12D, 18L:6D and 24L:0D.

Larvae from group 2 (11 dah) with inflated swimbladders (95% inflated, 4.63 ± 0.36 mm, mean ± S.D., $n = 20$) were drained from the 2000-l tank, homogeneously mixed in a 20-l tank and 200-ml randomly selected aliquots of larvae were transferred into each of 24 100-l tanks. There were 8 randomly selected replicate tanks per photoperiod treatment. Physical parameters were maintained: pH range, 7.6-7.8; DO_2 range, 8.7-8.8 mg l^{-1}; temperature range, 18.8-19.5°C; salinity range, 19.2-20.7‰; light intensity, 13.3 ± 2.4 μmol s^{-1} m^{-2} (mean ± S.D., $n = 24$). Stocking density was determined in a further 5 200-ml randomly selected aliquots of larvae (529 ± 83 larvae $tank^{-1}$; mean ± S.D.).

2.3.5. Larvae feeding and sampling

Larvae in Experiments 1 and 2 were fed enriched rotifers only (as described for rearing of group 2 larvae to 11 dah) at 0900 h and 1500 h each day to maintain a density of approximately 10 rotifers ml^{-1}.

In both experiments, a randomly selected sample of 10 larvae was collected from each tank at approximately 1300 h every 3 days and live larvae were observed under a dissecting microscope fitted with an ocular micrometer to determine measurement of TL (distance from the tip of the lower jaw to tip of the caudal fin), presence/absence of swimbladders, food in the gut and development of tail flexion.

In experiment 2, the final wet and dry weights of larvae were also measured in a randomly selected group of 10 larvae from each tank. Larvae were dried on blotting paper, placed onto a single, pre-weighed glass microscope slide and larvae weight determined to

the nearest 0.01 mg with an analytical balance ("Analytical Plus", Ohaus Corporation, Switzerland). The slides were then placed into a drying oven at 106°C for 16 h after which time each slide was weighed to the nearest 0.01 mg to estimate final larvae dry weight.

2.3.6. Daily water measurement

In both experiments, salinity, temperature, pH and DO_2 were measured daily using a water quality meter (Horiba U-10, Horiba Ltd, Japan). Total ammonia (Exp. 1 $\leq$ 0.6 mg l^{-1}; Exp. 2 $\leq$ 0.4 mg l^{-1}) was measured daily in at least one replicate tank from each treatment with a rapid test kit (E. Merck, Model 1.08024, Germany). Light intensity was measured with a light meter (LI-COR, model Li-1776, USA).

2.3.7. Statistical analyses

Data were assessed for homogeneity of variance using Cochran's test (C; Winer et al., 1991). For Experiment 1, data for final total length ($P < 0.00001$, $C = 0.9$) were heterogeneous and could not be transformed to satisfy the assumption of homogeneity of variance. For Experiment 2, data for survival were transformed by Log10 to satisfy the assumption of homogeneity. Experiments were designed for analysis using single factor analysis of variance (ANOVA). Where significant differences were found, means were compared by the Student-Newman-Keuls test (SNK). A posteriori power analyses of ANOVA of mean final total length and survival of snapper larvae in Experiment 2 were done to determine experimental power and number of replicates required to detect a range of minimum differences between means (Searcy-Bernal, 1994). Statistical analyses were conducted using Statgraphics Version 5.0 (STSC Inc., USA).

2.4. Results

2.4.1. Experiment 1: Effect of photoperiod from 3-15 dah

Unexplained mortality of larvae occurred in 4 of the 6 replicate tanks in the 18L:6D treatment soon after stocking. This resulted in low numbers of surviving larvae in 4 replicate tanks and by day 9 all larvae in these tanks had either died or were sampled.

Snapper larvae that were held in total darkness did not start feeding (Fig. 2.1) and were all dead 3-4 days after transfer (6-7 dah). Rotifers were not observed in the gut of sampled larvae held in 6L:18D until 9 dah but larvae held in 12L:12D, 18L:6D and 24L:0D had begun feeding by 6 dah and the number of larvae with rotifers in the gut was high and similar ($P > 0.05$) in these treatments (Fig. 2.1). Growth of larvae held in 6L:18D was slow (Fig. 2.2) and all larvae had died by 11 dah. Larvae held in 12L:12D, 18L:6D and 24L:0D continued growing for the duration of the experiment, however at 15 dah, the TL of larvae held in 18L:6D and 24L:0D, was similar ($P > 0.05$) and significantly greater ($P < 0.05$) than that of larvae held in 12L:12D.

The development of tail flexion was significantly affected by photoperiod ($P < 0.05$) (Fig. 2.3). Almost all (95%) larvae sampled from the 24L:0D treatment had commenced tail flexion by 12 dah. Alternatively, no larvae in the 12L:12D treatment and only 25% of larvae held in the 18L:6D treatment had commenced tail flexion by this time. By 15 dah, 95% and 48% of larvae had commenced tail flexion in the 18L:6D and 12L:12D treatments, respectively (Fig. 2.3).

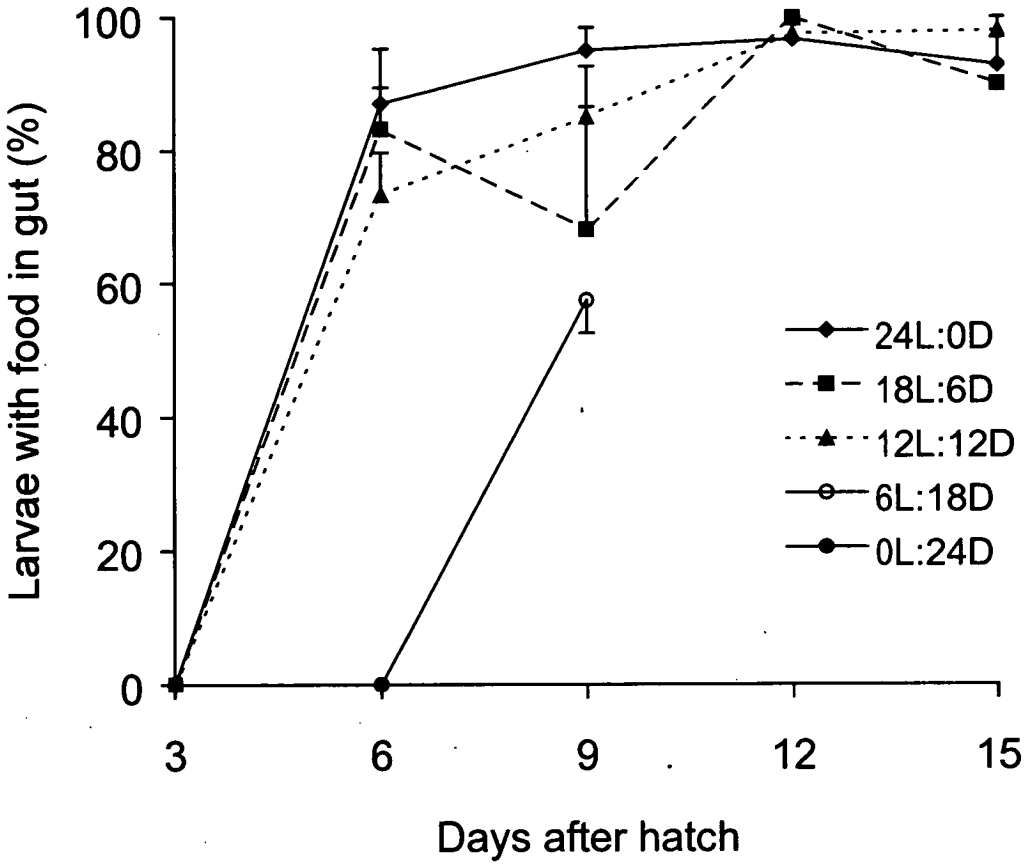

Fig. 2.1. Percentage of snapper larvae with rotifers in their gut grown from 3 dah to 15 dah in 100-l tanks under different photoperiod regimes. Data are means ± S.E. Experiment 1.

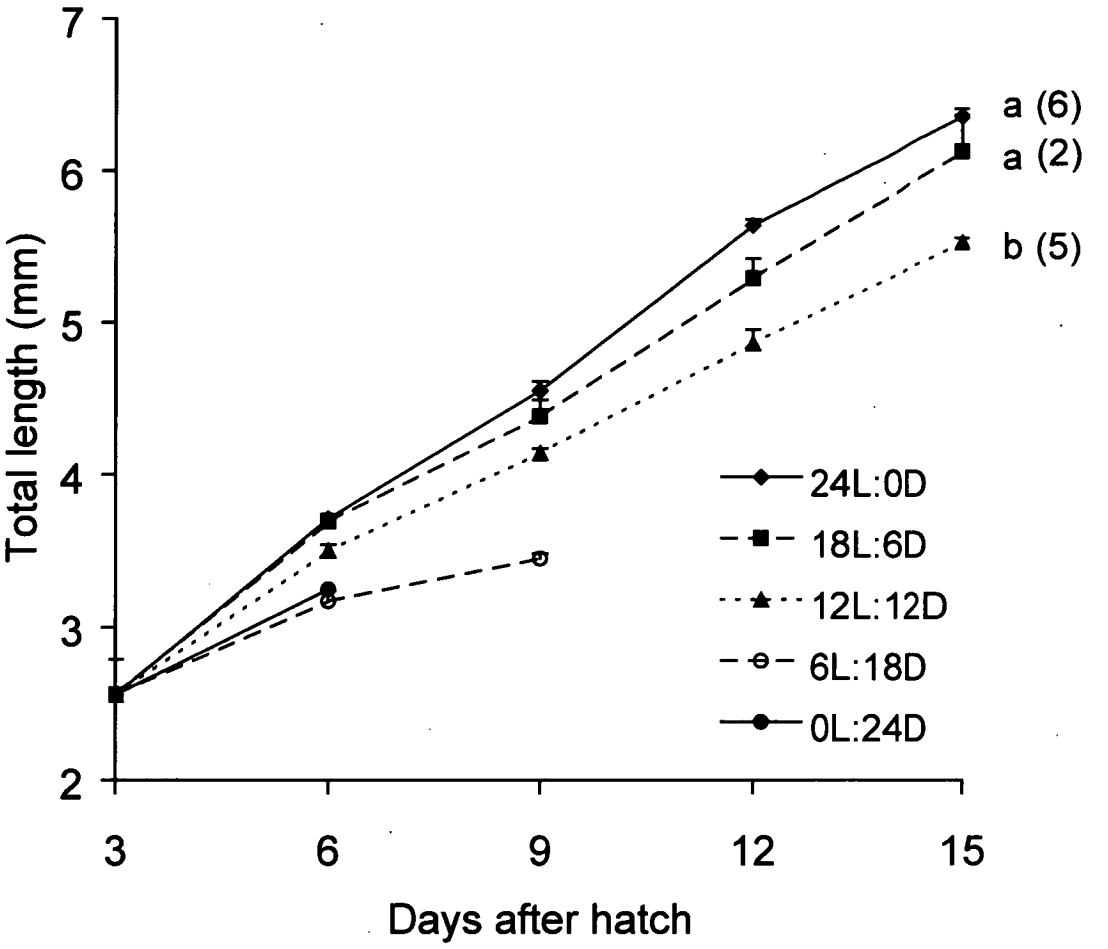

Fig. 2.2. Total length of snapper larvae grown from 3 dah to 15 dah in 100-l tanks under different photoperiod regimes. Data are means ± S.E. Points with different letters are significantly different ($P < 0.05$); (n) = number of tanks sampled. Experiment 1.

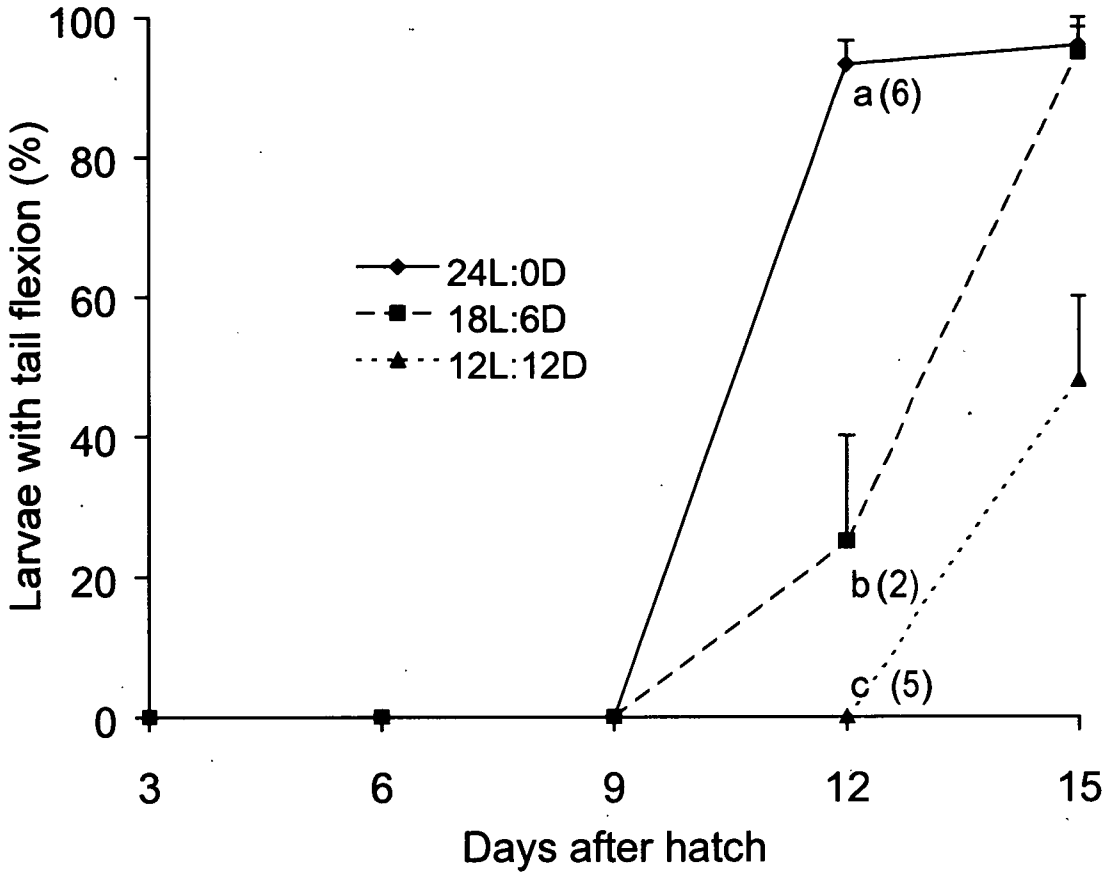

Fig. 2.3. Percentage of snapper larvae with tail flexion grown from 3 dah to 15 dah in 100-l tanks under different photoperiod regimes. Data are means ± S.E. Points with different letters are significantly different ($P < 0.05$); (n) = number of tanks sampled. Experiment 1.

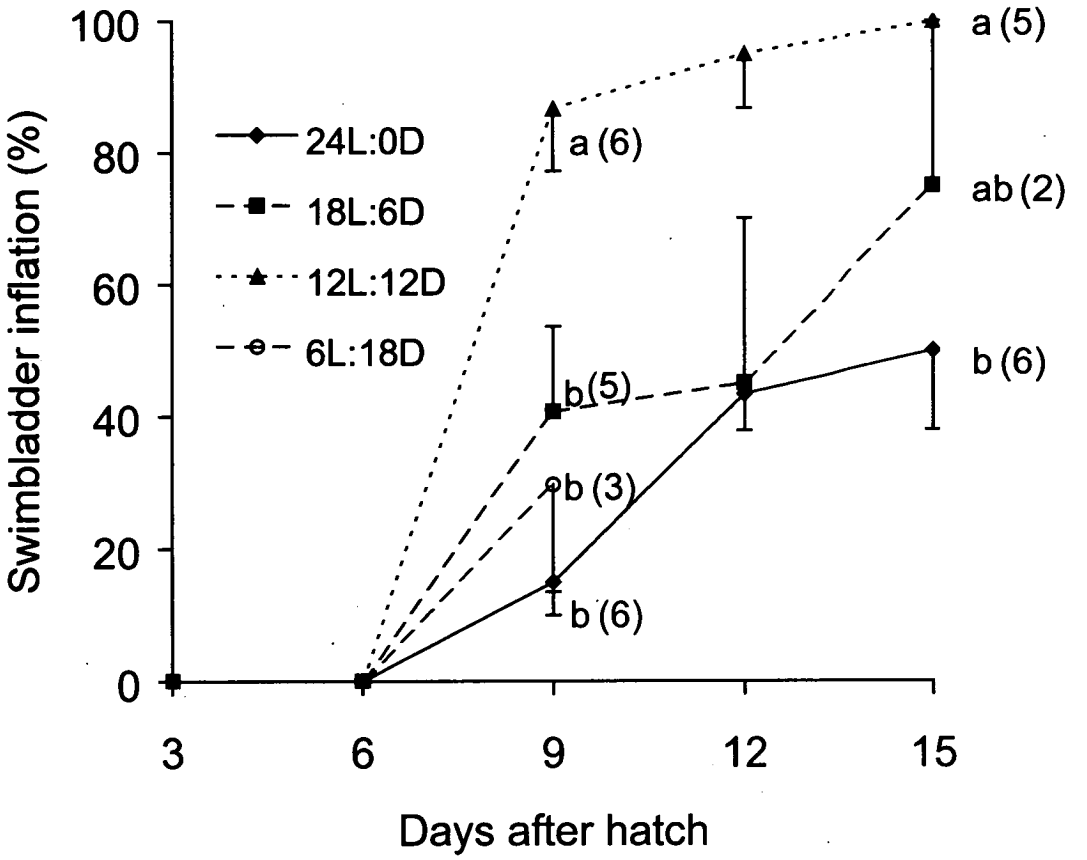

Fig. 2.4. Percentage of snapper larvae with inflated swimbladders grown from 3 dah to 15 dah in 100-l tanks under different photoperiod regimes. Data are means ± S.E. Points with different letters are significantly different ($P < 0.05$); (n) = number of tanks sampled. Experiment 1.

The number of larvae in the 12L:12D treatment with inflated swimbladders was significantly greater ($P < 0.05$) than all other treatments, which did not differ ($P > 0.05$) (Fig. 2.4). At 9 dah, almost 90% of larvae held in 12L:12D had inflated swimbladders, which was approximately 2, 3 and 6 times greater than larvae held in the 18L:6D, 6L:18D and 24L:0D treatments, respectively. At 15 dah, the number of larvae sampled with inflated swimbladders had increased in the 12L:12D, 18L:6D and 24L:0D treatments but multiple comparisons of means did not clearly identify differences due to insufficient replication (Fig. 2.4). Nevertheless, swimbladder inflation of larvae held in 12L:12D was 1.3 and 2.0 times greater than the 18L:6D and 24L:0D treatments.

2.4.2. Experiment 2: Effect of photoperiod from post swimbladder inflation

At day 6 of the experiment (17 dah), larvae in 1 replicate tank of the 24L:0D treatment had over-inflated swimbladders and began dying, and larvae in one replicate tank of the 18L:6D treatment were sucked out of the tank onto the mechanical filter due to failure of the internal mesh screen. Data from both tanks was discarded from the experimental analysis. Snapper larvae grew in all photoperiod treatments (Fig. 2.5), however after 6 d (17 dah), the TL of larvae held in the 18L:6D and 24L:0D treatments was significantly greater ($P < 0.05$) than that of larvae in the 12L:12D treatment. This trend continued and 32 dah larvae held in 18L:6D and 24L:0D, which did not differ significantly ($P > 0.05$) were approximately 18.3% and 15.0% longer, respectively, than larvae in 12L:12D (Fig. 2.5).

After 21 d (32 dah), the final wet and dry weights of larvae were significantly affected by photoperiod ($P < 0.05$) with the heaviest larvae in order of magnitude 18L:6D > 24L:0D > 12L:12D (Table 2.1). Larvae held in 18L:6D were approximately 1.3 and 1.9 times heavier than larvae held in 24L:0D and 12L:12D, respectively.

The ability to detect treatment differences varied greatly between the measured parameters of total length and wet or dry weights of snapper larvae. Significant differences between final mean wet weight ($F = 39.13$, $P < 0.05$) and dry weight ($F = 22.21$, $P < 0.05$) of snapper larvae were clearly identified, where 18L:6D > 24L:0D > 12L:12D. Analysis of mean data for final total length of snapper larvae, however, did not provide the same degree of sensitivity with $n = 7$ replicates. It was possible to detect a difference of 1.72 mm in mean final total length of larvae ($F = 12.26$, $P < 0.05$) between treatments (18L:6D and 12L:12D) with a high degree of experimental power (0.97); however, it was not possible to detect a significant difference ($P < 0.05$) of 0.29 mm which occurred between 18L:6D and 24L:0D treatments at final harvest. Power analysis showed that approximately 90 replicate tanks were required to detect a difference of 0.29 mm between the largest and smallest means with a power of 0.8 at $P = 0.05$ (Searcy-Bernal, 1994).

There was no difference in survival of larvae between photoperiod treatments ($P > 0.05$) (Table 2.1); however, statistical power analysis on ANOVA of number of surviving larvae showed that power of this experiment was low (0.1). At each time of sampling, the number of larvae with rotifers in their gut did not differ ($P > 0.05$) between photoperiod treatments and ranged from approximately 70-100%.

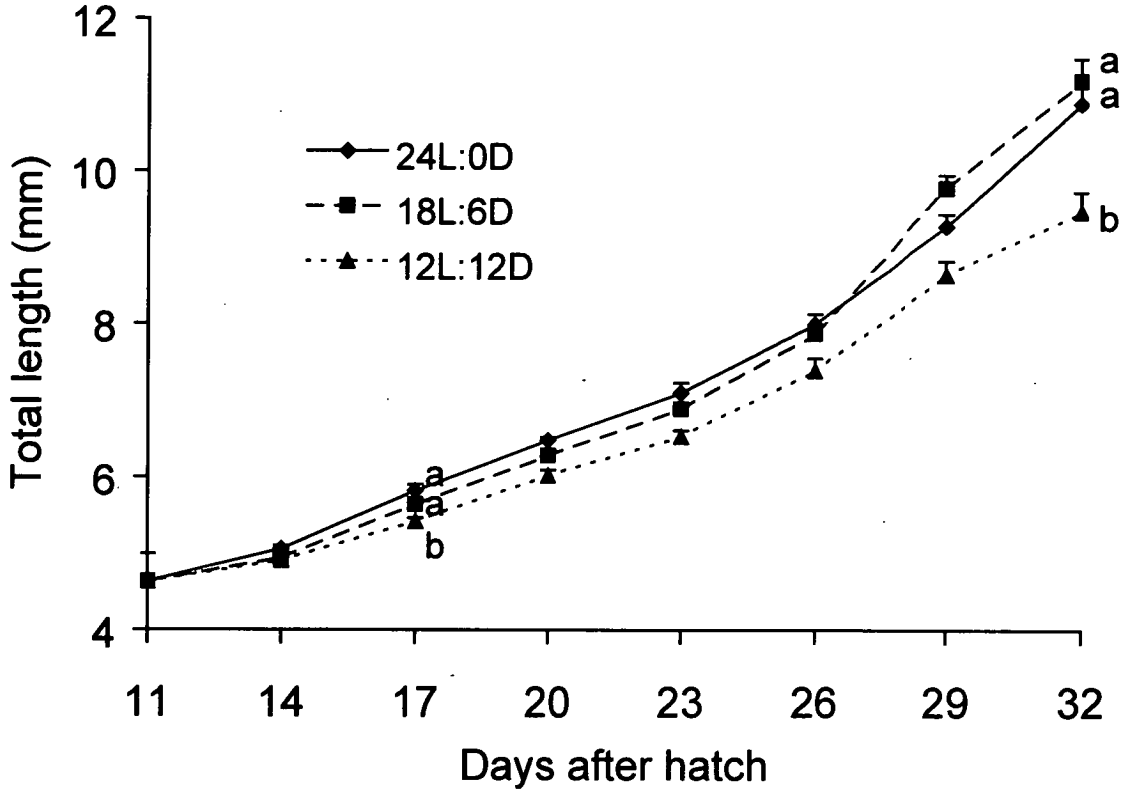

Fig. 2.5. Total length of snapper larvae grown from 11 dah to 32 dah in 100-l tanks under different photoperiod regimes. Data are means ± S.E. (n = 8 for day 3; n = 7 from day 6 onwards). Points with different letters are significantly different ($P < 0.05$). Experiment 2.

Table 2.1. Final harvest survival, and wet and dry weights of snapper larvae grown From 11 dah to 32 dah in 100-l tanks under different photoperiod regimes.

Experiment 2.

Photoperiod regime	Survival (%)	Final wet weight (mg)	Final dry weight (mg)
12L:12D	16.6 ± 6.9 a	8.48 ± 0.75 c	1.53 ± 0.18 c
18L:6D	11.3 ± 2.1 a	16.26 ± 0.53 a	2.76 ± 0.1 a
24L:0D	14.8 ± 3.0 a	12.63 ± 0.56 b	2.12 ± 0.1 b

Data are means ± S.E. for $n = 7$ tanks. Means within columns with different letters Are significantly different ($P < 0.05$).

2.5. Discussion

Photoperiod clearly influenced the growth and development of snapper larvae; however, optimal photoperiod for culture of snapper varied with ontogeny. In Experiment 1, snapper larvae held in constant darkness did not commence feeding and died within 3-4 days (6-7 dah) after the onset of the experiment. This confirms that snapper, like most marine teleosts are visual feeders (Blaxter, 1986; Pankhurst, 1994; Pankhurst and Hilder, 1998; Cox and Pankhurst, 2000), which is a sensory process that requires light. Our results indicate that larvae held in darkness died due to starvation and confirms an earlier report of the time to starvation in this species when denied food (Pankhurst et al., 1991).

A short light phase (6L:18D) retarded feeding onset in young larvae by 3 d compared to other treatments (12L:12D, 18L:6D and 24L:0D). While temperature has a potent effect on the rate of development of fish larvae (Blaxter, 1969; Blaxter, 1988; Youson, 1988) it appears that development (in this case feeding onset) has been dissociated from temperature control in the 6L:18D larvae compared to other treatments. That is, despite effect of temperature, development was retarded. This supports earlier reports that, physical factors besides temperature affect rate of larval development (Blaxter, 1969; Tandler and Helps, 1985).

Growth (total length) of larvae 3-15 dah was significantly greater in longer light phases of 18L:6D and 24L:0D, than the 12L:12D treatment. This very likely reflects the benefits of increased duration of the visual feeding period (Tandler and Helps, 1985; Howell et al., 1998; Boeuf and Le Bail, 1999). Also, in contrast to the retarding effect of a short light phase (6L:18D) on onset of larval feeding, a longer light phase accelerated larval development (as defined by the developmental milestone of tail flexion) such that 95% of 24L:0D larvae commenced tail flexion by day 9 (12 dah) compared with 95% and 48% of larvae having commenced tail flexion in 18L:6D and 12L:12D treatments after 12 days (15

dah), respectively. Despite some intraspecific variation, most fish species must reach a certain size before they can metamorphose into a juvenile (Youson, 1988). Length and (or growth rate) of bony fish larvae is one of the most important factors that determines development and the onset of metamorphosis (Youson, 1988). In a study similar to ours, Tandler and Helps (1985) demonstrated that photoperiod had a positive effect on growth rate of *S. aurata* larvae; that is, larvae grown under an extended light phase (24L:0D) were much larger (more developed) than larvae of the same age grown in a short light phase (12L:12D).

Initial swimbladder inflation, on the other hand, was highest when light was provided at the shorter light phase of 12L:12D. Successful swimbladder inflation is necessary for subsequent growth and survival of cultured physoclistous larvae (Battaglene, 1995). Failure to inflate functional swimbladders can result in reduced larval growth (Battaglene and Talbot, 1990, 1992), lordotic spinal deformities (Takashima, 1978; Chatain, 1994; Kitajima et al., 1994) and high mortality (Spectorova and Doroshev, 1976; Chatain, 1986, 1987; Chapman et al., 1988; Chatain and Dewavrin, 1989). Despite the potential benefits of increased length growth in larvae 3-15 dah at the longer light: shorter dark phases of 24L:0D and 18L:6D, these are likely offset by the potential benefits of high rates of swimbladder inflation at 12L:12D. The latter is supported by the observation that cultured fish without swimbladders fail to thrive (Battaglene, 1995) and as a result methods have been developed to remove these fish from culture (Chatain and Corrao, 1992). Accordingly, we suggest a photoperiod of 12L:12D to be optimal for culture of snapper during this early period of development.

In Experiment 2, growth (total length) of post-swimbladder inflated snapper larvae (11 dah) larvae in 18L:6D and 24L:0D did not differ but both treatments had a greater total length than larvae from the 12L:12D treatment. The heaviest larvae at experiment

termination were in the 18L:6D treatment. There was no difference in survival of larvae between photoperiod treatments; however, statistical power of the experiment (Searcy-Bernal, 1994) was very low (0.1) as the high variability within treatments meant that replication was inadequate. It would therefore be imprudent to accept the null hypothesis that photoperiod did not affect survival of snapper larvae, and further research is warranted to determine optimal photoperiod for survival.

In general, long photoperiod improves larval rearing quality due to the 'synergistic effect of food availability and light' (Boeuf and Le Bail, 1999); larvae, being sight feeders, are able to feed for longer periods. However, optimal photoperiods are species-specific and maximum hours of feeding is not necessarily the most important factor of optimal conditions (Blaxter, 1986). For example, extended to continuous photoperiods increased growth of 20 dah larvae of greenback flounder, *Rhombosolea tapirina* (18 = 24 h > 12 h > 6 h light), but photoperiod from 6-24 h did not affect survival. (Hart et al., 1996). Fuchs (1978) found that growth of sole, *Solea solea* larvae was greater at 18 and 24 h light than at 12 h light, but survival was not affected by photoperiod. Similarly, growth of 8-20 dah barramundi, *Lates calcarifer* larvae was better at extended photoperiods (16 = 24 h > 8 h light) but survival was not affected by photoperiod (Barlow et al., 1995). For the sparid, *S. aurata*, growth of larvae was greatest in continuous light of approximately 1,000-3,500 lx; however, survival was best at an intermediate photoperiod of 15 h light (Peguin, 1984, cited in Tandler, 1993; Tandler and Helps, 1985). Survival of *D. labrax* was also best in a 12 h photoperiod, but like snapper in this study, larval growth in an 18 h photoperiod was greater than in 12 or 24 h photoperiods (Barahona-Fernandes, 1979).

Larvae are continuously active in daylight in rearing tanks and this is associated with searching for food (Blaxter, 1986). Therefore, a longer light phase provides longer feeding duration but also extends the duration of the foraging (searching) behaviour such that a

greater weight gain at 18L:6D compared to 24L:0D may simply reflect the energy budget. Japanese red sea bream (*P. major*) larvae, a proposed subspecies of *P. auratus* (Tabata and Taniguchi, 2000), undergo diurnal fluctuation in their swimbladder volume (Kitajima et al., 1985; Kitajima et al., 1993). During the day, when larvae are active and expending energy (Blaxter, 1986), volume of swimbladders is reduced; however, at night when larvae are not actively swimming (conserving energy), the swimbladders are inflated to maintain neutral buoyancy (Kitajima et al., 1993). There is a paucity of information relating to diurnal change in swimbladder volume of snapper larvae. However, Pankhurst et al. (1991) observed that snapper larvae become inactive during the dark phase, suggesting that changes in swimbladder volume may be similar to red sea bream. Therefore, accrual of conserved energy during a low activity 'dark' phase (18L:6D) may exceed the benefit of an extended but active feeding duration (24L:0D).

In addition, fish larvae tend to increase consumption of feed as prey density (availability) increases (Kamler, 1992); however, in culture conditions where prey density is maintained at artificially high levels, consumption rates can be so high and assimilation rates so low (through rapid gut evacuation time) that growth is reduced (Howell et al., 1998). In our study, prey densities ranged from 1-2 ml^{-1} in the morning prior to feeding, to 10 ml^{-1} after feeding. This range of prey density is considered high and indeed Parra and Yúfera (2000) demonstrated that growth of 15 dah gilthead seabream (*Sparus aurata*) larvae was the same when fed prey at 1 ml^{-1} and 10 ml^{-1}. It is possible that continual feeding (24L:0D) of snapper larvae resulted in suboptimal assimilation and therefore reduced growth compared to that in 18L:6D, where larvae did not feed during the 'dark' phase.

Alternatively, snapper larvae may have displayed a circadian feeding pattern. This was demonstrated for *D. labrax* larvae, which remained actively swimming but had a marked

reduction in feeding activity from 0000 h to 0800 h (Ronzani Cerquiera and Chatain, 1991). Snapper larvae in continuous light (24L:0D) may have remained active but did not feed for a period of time, thus expending energy swimming rather than for growth. Snapper larvae in the 18L:6D treatment had a dark phase from 0200 to 0800 h, which may have coincided with the natural non-feeding rhythm.

Clearly, differences in wet and dry weights of 32 dah snapper larvae are large compared to corresponding differences in total length of larvae. Snapper larvae undergo metamorphosis from about 25-35 dah (Battaglene, 1992) and it is most likely that biomass of fish is increasing due to development of internal organs (Foscarini, 1988), vertebral column and caudal skeleton (Matsuoka, 1982), myotomal musculature (Matsuoka and Iwai, 1984; Pankhurst et al., 1991), fins (Fukuhara, 1976; cited in Foscarini, 1988; Pankhurst et al., 1991) and scales (Battaglene, 1992). Therefore estimate of wet and/or dry weights of 32 dah larvae provides a more sensitive evaluation of treatment effect than comparison of larval total length.

2.6. Conclusion

Optimal photoperiod for growth and development of snapper larvae in culture varied with life stage. For first-feeding snapper larvae, although growth increased as photoperiod was increased, swimbladder inflation was best at 12L:12D. Once larvae had inflated their swimbladders, growth of larvae was greatest at a 18L:6D photoperiod. Survival of post-swimbladder inflated snapper larvae was not affected significantly by photoperiod. However, as the power of the experiment to detect treatment effects on survival was very low further research is warranted to determine the effects of photoperiod on survival of snapper larvae. The photoperiod regime for optimal swimbladder inflation and growth to

metamorphosis was: 12L:12D from first-feed to swimbladder inflation; 18L:6D post swimbladder inflation to metamorphosis.

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CHAPTER 3

The effects of salinity and temperature on growth and survival of Australian snapper, *Pagrus auratus* larvae

3. The effects of salinity and temperature on growth and survival of Australian snapper, *Pagrus auratus* larvae

3.1. Summary

The effects of salinity and temperature on performance of first-feeding to pre-metamorphosis were determined on Australian snapper, *Pagrus auratus*, larvae held in 100-l recirculation tanks. Feeding onset, survival, growth and initial swimbladder inflation were assessed initially after transfer from 35‰ at eight salinity treatments (5, 10, 15, 20, 25 30, 35 and 45‰) in larvae from 3-21 days after hatching (dah). Optimal salinity for larval survival was not clearly identified, however survival was best within the range of 20‰ to 35‰. Larval growth was similar within the range of 10‰ to 35‰ (6.8 - 7.1 mm total length [TL]) but larvae were 15% shorter at 45‰. Final swimbladder inflation of larvae was not affected by salinity and ranged from 52.2% to 86.7%, however initial swimbladder inflation was delayed in 10‰ and 45‰. The presence of calculi in the urinary bladder of larvae was positively correlated with increasing salinity but no relationship between urinary calculi and larval survival was observed. Larvae in all salinity treatments (except 5‰) started feeding at the same time and the number of larvae with food in the gut was the same for all treatments (70-100%) during the experiment. In a second experiment, feeding onset, survival, growth and initial swimbladder inflation were assessed after transfer from 21°C at seven temperature treatments (15, 18, 21, 24, 27, 30 and 33°C) in larvae from 3-21 dah. All larvae transferred from 21°C to 30°C and 33°C, and 27°C, died after 3 and 9 days, respectively. Survival was not significantly different between 15°C and 24°C but power of the experiment to detect effects on survival was low (0.24) due to high variability within treatments. Larval growth increased as temperature was increased; larvae at 24°C (4.8 ± 0.2 mg wet weight) were 6-fold heavier than larvae at

15°C. Larvae grown at 18°C, 21°C and 24°C inflated their swimbladders at high and similar rates. However, larvae grown in 15°C and 27°C inflated their swimbladders at significantly slower rates than those grown in 18°C to 24°C. The incidence of urinary calculi occurred earlier and in a greater number of larvae when temperature was increased. No difference in the number of larvae with food in their guts was recorded at any time of sampling during the experiment. In a third experiment, feeding onset, survival, growth and initial swimbladder inflation were assessed at combinations of two salinities (20‰ and 35‰) and three temperatures (18°C, 21°C, and 24°C) in larvae from 3-24 dah. Survival of snapper larvae was not significantly different between treatments, however power of the experiment was low (0.1) due to high variability within treatments. Growth was not affected by salinity but larvae increased in size as temperature was increased and there was no interaction of salinity and temperature. Larvae in all treatments commenced feeding and inflated their swimbladders at similar rates in all treatments. Salinity and temperature influenced the incidence of urinary calculi and there was an interaction between the parameters. Based on our results in terms of larval performance (growth), development and survival we conclude that the optimal conditions for larval rearing of snapper from first-feeding (3 dah) to pre-metamorphosis (24 dah) are combinations of salinity from 20‰ to 35‰ and a temperature of 24°C.

3.2. Introduction

Optimising survival and growth of larvae is often essential for successful intensive culture of fish (Tandler et al., 1989). Performance of larvae is influenced by a combination of biotic and abiotic factors (Battaglene, 1995; Hart et al., 1996). Two of the most potent abiotic factors in the life of marine and brackishwater organisms are temperature and salinity (Kinne, 1963). The eggs and larvae of many marine fish are euryhaline and eurythermic (Battaglene, 1995), however the tolerance of larvae to combinations of salinity and temperature is species specific and may change during ontogeny, and be influenced by maternal environmental conditions (Blaxter, 1969; Alderdice, 1988; Howell et al., 1998).

Salinity can affect efficiency of yolk utilisation and larval growth rate and survival by influencing the amount of energy needed for osmoregulation (Howell et al., 1998). Buoyancy of eggs and larvae can also be affected by salinity, which can influence the ability of larvae to swim to the water surface to gulp air and inflate their swimbladders (Hadley et al., 1987; Battaglene and Talbot, 1990, 1993). Failure to inflate swimbladders can result in larval mortality (Chatain, 1986, 1987; Chapman et al., 1988; Chatain and Dewavrin, 1989), reduced larval growth (Battaglene and Talbot, 1990, 1992), skeletal deformities (Chatain, 1994; Kitajima et al., 1994; Trotter et al., 2001) and has sometimes been associated with formation of abnormal urinary calculi (Yamashita, 1971; Modica et al., 1993).

Calculi, composed principally of calcium phosphate (Sakai et al., 1996), have been observed in the urinary bladder and kidney of larvae and juveniles of several fish species including seabream (Favaloro and Mazzola, 2000), snapper (Battaglene, 1995), Japanese flounder (Sakai et al., 1996) and European sea bass (Menu et al., 1998). The causes of formation of urinary calculi are unknown, however it may be a pathological problem (Modica et al., 1993) or associated with specific larval rearing techniques and hatchery

sites (Menu et al., 1998). Despite their abnormal presence, urinary calculi are often not associated with increased larval mortality or a reduction in larval growth (Battaglene, 1995; Sakai et al., 1996; Favaloro and Mazzola, 2000).

Temperature usually has a greater effect on fish performance than salinity (Rombough, 1996). Temperature can affect virtually all aspects of fish reproduction (van Der Kraak and Pankhurst, 1996) and larval development such as hatching size, efficiency of yolk utilisation, growth, feeding rate, time to metamorphosis, digestion rates and metabolic demand (Blaxter, 1988; Rombough, 1996). However, due to the interactive effects of salinity and temperature on osmoregulation they should be considered together when determining optimal conditions for tolerance and performance (Kinne, 1963).

Many studies have dealt with short-term effects of salinity and temperature on marine fish eggs, larvae and juveniles (e.g. Kinne, 1963; Holliday, 1969; Freddi et al., 1981; Rombough, 1996). However, the effect of long-term exposure to different salinities and temperature on marine fish larval performance has been little studied (Hart et al., 1996). Such studies are essential to determine optimal salinity and temperature for larval rearing (Tandler et al., 1995; Hart et al., 1996).

Australian snapper, *Pagrus auratus*, is an important commercial and recreational species found in Australian and New Zealand waters (Bell et al., 1991; Battaglene and Bell, 1991; Pankhurst et al., 1991). While wild catches are declining in Australia (ABARE, 2000) aquaculture of snapper is increasing in Australia using intensive larval rearing techniques followed by growout in sea-cages (Battaglene and Talbot, 1992; Battaglene and Fielder, 1997; Fielder et al., 2002, Chapter 2). These hatchery techniques are similar to those used for culture of the closely related Japanese red sea bream, *P. major* (Foscarini, 1988) which is a proposed sub-species of *P. auratus* (Tabata and Taniguchi, 2000).

The environmental conditions in which Australian snapper larvae are typically reared, are principally based on ambient coastal conditions during the natural spring/summer spawning season (Battaglene and Talbot, 1992). Salinity and temperature during this time are approximately 35‰ and 16-23°C, respectively (Pankhurst et al., 1991; Battaglene, 1995). However, the salinity and temperature combination for optimal development, growth, and survival of snapper larvae is unknown.

Similarly, despite major aquaculture production of red sea bream in Japan, relatively little is known about the optimal environmental requirements for larval rearing of this fish (Mihelakakis and Yoshimatsu, 1998). To date, research has focussed on effects of salinity and temperature on embryonic development (Matsuura and Kakuda, 1980), hatching (Apostolopoulos, 1976) and incubation period, hatching rate and morphology of newly hatched larvae (Mihelakakis and Yoshimatsu, 1998).

The aim of this study was to investigate the effects of salinity and temperature on larval snapper ontogeny to determine the optimal salinity and temperature protocol for swimbladder inflation, growth and survival of snapper larvae reared in tanks.

3.3. Materials and Methods

Three experiments were done at the NSW Fisheries Port Stephens Fisheries Centre from May 1998 to February 1999.

3.3.1. Source of larvae

Fertilised eggs were obtained from first generation hatchery-reared broodstock held in a 17,000-l flat-bottomed tank, that was part of an independent, recirculating system operated with an external 700-l egg collection sump, pump and mechanical and biological filters. The tank was held in a temperature and photoperiod controlled room.

Three groups of snapper larvae (group 1, 2 and 3) were produced on independent occasions after two female snapper (range 1.4-2.9 kg) were induced to spawn on each occasion using intramuscular pellet implants containing LHRHa (des-gly^{10}, D-trp^{6}, pro^{9}-ethylamide; Peptech Animal Health Pty Limited, Australia) (range 100-207 μg kg^{-1}). Salinity and temperature of water when spawning occurred were similar for each larval group: group 1 – 34.5‰ and 19.6°C; group 2 – 29.0‰ and 18.5°C; group 3 – 30‰ and 20.7°C. Fertilised eggs were transferred from the collection sump to a 2,000-l conical bottom tank (group 1) and 750-l flat-bottomed tanks (groups 2 and 3) which were filled with sterilised seawater (20 mg l^{-1} sodium hypochlorite for 24 h then neutralised with sodium thiosulphate) for incubation under darkness, salinity and temperature of 35.3‰ and 21 ± 1°C (group 1), 31.0‰ and 21 ± 1°C (group 2) and 35.9‰ and 20.5 ± 1°C (group 3). For group 1 larvae, tank water was recirculated through a mechanical and biological filter at 5 l min^{-1}. For group 2 and 3 larvae, tank water was kept static and about 50% of the tank volume was drained daily and replaced with sterilised seawater. All tanks were gently aerated (~200 ml min^{-1}) and were siphoned daily to remove unhatched eggs and detritus. Larvae in all groups were kept in the incubation tanks for 3 days after hatching (dah).

3.3.2. Tanks used for salinity and temperature experiments

Experiments were conducted in 100-l tanks described by Fielder and Bardsley (1999). Each tank was provided with surface skimmers to remove surface films. Incandescent lighting (Osram Halogen Decostar 51 12 V 50 W) was provided overhead on a 14:10 h light:dark cycle. For Experiments 2 and 3, each tank had a submersible aquarium heater (200 W). Each tank was enclosed within a box made from black plastic sheet to prevent reflected light affecting surrounding tanks. Approximately 5% of the water in each tank was exchanged each day and salinity was maintained by adding rain water when needed.

3.3.3. Experiment 1: Effect of salinity from 3-21 dah

The aim of the experiment was to determine the effect of salinity on growth and development of snapper larvae from first-feeding (3 dah) to 21 dah. The salinity treatments were 45, 35, 30, 25, 20, 15, 10, and 5‰.

Larvae from group 1 (3 dah; 3.4 ± 0.1 mm total length [TL]; mean ± S.D., n = 20) were harvested from the 2,000-l tank and transferred to a 20-l bucket in which they were mixed homogeneously by slowly raising and lowering a perforated 27-cm diameter plastic disc. Three randomly selected 50-ml samples of larvae were transferred into each of the 32 experiment tanks filled with seawater (34.5 ± 0.6‰; 20.5 ± 0.3°C). Four randomly selected replicate tanks were used per salinity treatment. Physical tank parameters were maintained: pH range, 7.8-8.4; dissolved oxygen (DO_2) > 5.0 mg l^{-1}; temperature range, 20.2-22.7°C; total ammonia-nitrogen (< 0.6 mg l^{-1}); light intensity, 8.6 ± 0.9 µmol s^{-1} m^{-2} (mean ± S.D., n = 32). Five 150-ml samples (3 x 50 ml) of larvae were taken to estimate initial stocking densities (1576 ± 136 larvae $tank^{-1}$; mean ± S.D.).

After stocking, salinity treatments were imposed by adding rainwater (30‰ to 5‰ treatments) or artificial seasalt solution (100 g l^{-1}; Instant Ocean®, Aquarium Systems Inc., USA) (45‰ treatment). Salinity in treatment tanks was changed from 35‰ to 45‰, 30‰, 25‰, 20‰, 15‰, 10‰, and 5‰ in 14.7 ± 0.3 h, 5.2 ± 0.2 h, 7.3 ± 0.7 h, 8.5 ± 0.5 h, 10.7 ± 1.7 h, 13.3 ± 1.2 h, and 16.9 ± 0.9 h, respectively.

3.3.4. Experiment 2: Effect of temperature from 3-21 dah

The aim of the experiment was to determine the effect of temperature on growth and development of snapper larvae from first-feeding (3 dah) to 21 dah. The temperature treatments were: 33°C, 30°C, 27°C, 24°C, 21°C, 18°C and 15°C.

Larvae from group 2 (3.3 ± 0.2 mm TL; mean ± S.D., n = 20) were harvested in the same way as that described for Experiment 1 and were stocked into each of 28 experiment tanks (30.6 ± 0.5‰; 20.3 ± 0.3°C), which were managed similarly to those described in Experiment 1. Four randomly selected replicate tanks were assigned per temperature treatment. Physical tank parameters were maintained: pH range, 7.7-8.0; DO_2 > 7.0 mg l^{-1}; salinity range, 29.4-34.0‰; total ammonia-nitrogen (< 0.3 mg l^{-1}); light intensity, 13.4 ± 3.9 µmol s^{-1} m^{-2} (mean ± S.D., n = 28). The data presented for salinity (29.4-34.0‰) is the range of salinity recorded in all of the tanks during the experiment. Although attempts were made to maintain salinity in treatment tanks at 30‰ some variation (increase in salinity) occurred due to evaporation. The degree of evaporation increased as treatment temperature was increased, thus some of the salinities were higher than the target 30‰. Three 150-ml samples (3 x 50 ml) of larvae were taken to estimate initial stocking densities (627 ± 31 larvae $tank^{-1}$; mean ± S.D.).

After stocking, temperature treatments were imposed by reducing ambient air temperature to approximately 14°C and activating submerged heaters. Temperature in treatment tanks was changed from 21°C to within 1.0°C of 33°C, 30°C, 27°C, 24°C, 18°C and 15°C in 31.7 ± 9.0 h, 7.6 ± 0.6 h, 7.4 ± 0.6 h, 5.6 ± 0.8 h, 36.2 ± 10.5 h, and 45.3 ± 0.0 h, respectively.

3.3.5. Experiment 3. Effect of interaction of salinity and temperature from 3-24 dah.

The aim of the experiment was to determine the interaction of salinity and temperature on growth and development of snapper larvae from first-feeding (3 dah) to 24 dah. Based on results from Experiments 1 and 2, two salinity (20‰ and 35‰) and three temperature (18°C, 21°C, and 24°C) treatments were tested in an experiment designed for factorial analysis.

Larvae from group 3 (3.5 ± 0.2 mm TL; mean ± S.D., n = 20) were harvested in the same way as that described for Experiment 1 and were stocked into each of 30 experiment tanks (35.3 ± 0.2‰; 20.2 ± 0.2°C), which were managed similarly to those described in Experiment 1. Five randomly selected replicate tanks were allocated for each salinity/temperature treatment. Physical tank parameters were maintained: pH range, 7.4-7.9; DO_2 > 7.0 mg l^{-1}; salinity range, 19.8-21.2‰ (target 20‰), 34.7-36.7 (target 35‰); temperature range, 18.4-19.1°C (target 18°C), 20.8-21.4°C (target 21°C), 22.9-23.8°C (target 24°C); total ammonia-nitrogen (< 0.6 mg l^{-1}); light intensity, 15.3 ± 4.9 µmol s^{-1} m^{-2} (mean ± S.D., n = 32). Five 150-ml samples (3 x 50 ml) of larvae were taken to estimate initial stocking densities (896 ± 154 larvae $tank^{-1}$; mean ± S.D.).

After stocking, salinity and temperature treatments were imposed by reducing ambient air temperature to approximately 14°C and activating submerged heaters. Salinity in treatment tanks was changed from 35‰ to 20‰ by adding rainwater over a period of 7.5 h. Temperature in treatment tanks was changed from 21°C to within 1.0°C of 24°C and 18°C in 24 h and 9 h, respectively.

3.3.6. Larvae feeding and sampling

Larvae in all experiments were fed enriched rotifers (size range 160-260 µm lorica length) (as described in Chapter 2) at 0900 h and 1500 h each day to maintain a density of

approximately 10 rotifers ml^{-1}. Transfer shock of rotifers due to different rotifer culture and treatment tank salinity and temperature conditions was reduced (Fielder et al., 2000) by adding rotifers directly from a culture tank with salinity and temperature most similar to that of the treatment tank. In Experiment 1, rotifers were transferred from 15‰ into 5-15‰ and from 25‰ into 20-45‰. In Experiment 2, rotifers were transferred from 15°C into 15-21°C and from 24°C into 24-33°C. In Experiment 3, rotifers were transferred from a culture tank at 20‰ and 18°C into treatment combinations of 20‰ and 18, 21 and 24°C; and from a culture tank at 35‰ and 18°C into treatment combinations of 35‰ and 18, 21 and 35°C.

In all experiments, a randomly selected sample of 10 larvae was collected by beaker from each tank at approximately 1300 h every 3 days and live larvae were observed under a dissecting microscope fitted with an ocular micrometer to measure total length (TL = distance from the tip of the lower jaw to tip of the caudal fin), and to determine presence/absence of inflated swimbladders, food in the gut, urinary calculi and development of tail flexion.

In all experiments, the final wet and dry weights of larvae were also measured in a randomly selected group of 6 to 10 larvae from each tank. Larvae were dried on blotting paper, placed onto a single, pre-weighed glass microscope slide and larvae weight was determined to the nearest 0.01 mg with an analytical balance ("Analytical Plus", Ohaus Corporation, Switzerland). The slides were then placed into a drying oven at 106°C for 16 h after which time each slide was cooled in a dessicator and weighed to the nearest 0.01 mg to estimate final larvae dry weight.

3.3.7. Daily water measurement

Salinity, temperature, pH and DO_2 were measured daily to the nearest 0.1‰, 0.1°C, 0.1 pH unit and 0.1 mg l^{-1}, respectively using a water quality meter (Horiba U-10, Horiba Ltd,

Japan). Total ammonia was measured daily in at least one replicate tank from each treatment with a rapid test kit (E. Merck, Model 1.08024, Germany). Light intensity was measured to the nearest 0.1 μmol s^{-1} m^{-2} with a light meter (LI-COR, model Li-1776, USA).

3.3.8. Statistical analyses

Experiments 1 and 2 were designed for analysis using single factor analysis of variance (ANOVA) and Experiment 3 was designed for analysis using two factor ANOVA (salinity and temperature were both fixed factors). Data were assessed for homogeneity of variance using Cochran's test (*C*; Winer et al., 1991) and where necessary, data were transformed (percent survival by log (x), (Experiments 1 and 2) to satisfy the assumption of homogeneity of variance. Cochran's tests after transformation were not significant. It was not possible to achieve homogeneity of the number of urinary calculi in Experiments 1, 2 and 3. Where significant differences were found by ANOVA in Experiments 1 and 2, means were compared by the Student-Newman-Keuls test (SNK). Data for final survival and mean final presence of urinary calculi, and mean final presence of urinary calculi and salinity in Experiment 1 were compared by regression analyses. Where significant interactions between factors occurred ($P < 0.05$) in Experiment 3, for each level of one factor, multiple comparison of the means of the other factor was performed using SNK. Where there was no significant interaction between factors ($P > 0.05$) multiple comparison of means of each main effect was done using SNK. A posteriori power analysis of ANOVA of mean swimbladder inflation of 12 dah larvae (Experiment 1) and mean final survival of snapper larvae (Experiments 2 and 3) was done to determine experimental power (Searcy-Bernal, 1994). Statistical analyses were done using Statgraphics Version 5.0 (STSC Inc., USA).

3.4. Results

3.4.1. Effect of salinity from 3-21 dah

Salinity had a significant effect ($P < 0.05$) on survival and growth of snapper larvae. All larvae held in 5‰ died within 48 h of transfer from 35‰ to 5‰. Some larvae survived for 18 days in all salinities from 10‰ to 45‰ (Table 3.1). Multiple comparison of means failed to identify clearly the optimal salinity for larval survival, however best survival was achieved in the salinity range of 20‰ to 35‰. Larvae were significantly shorter ($P < 0.05$) at 45‰ than at salinities ranging from 10‰ to 35‰, which did not differ significantly. Similarly, final wet weight and dry weight of larvae did not differ significantly when grown at salinities ranging from 15‰ to 35‰ ($P > 0.05$). Salinity had a significant effect ($P < 0.05$) on presence of urinary calculi in larvae (Table 3.1). The incidence of calculi was related strongly to salinity (Fig. 3.1). In general, the number of larvae with calculi increased as salinity was increased. After 18 days the number of larvae grown in salinities between 10‰ to 25‰ with calculi was low and did not differ significantly, however most larvae grown in salinities from 30‰ to 45‰ had developed calculi. Incidence of urinary calculi was not related to survival of larvae. Salinity did not affect final swimbladder inflation ($P > 0.05$), which ranged from 52.2% to 86.7% of larvae (Table 3.1). However, the rate of initial swimbladder inflation was significantly affected by salinity ($P < 0.05$) (Fig. 3.2). The main treatment effects were not clearly identified, but by 9 dah, trends in data show that larvae grown at salinities from 15‰ to 35‰ had inflated their swimbladders at high and similar rates, whereas larvae grown in salinities of 10‰ and 45‰ had low incidence of swimbladder inflation. By 12 dah, swimbladder inflation did not differ between treatments ($P < 0.05$) but power of the experiment was low (0.4) due to

high variability within treatments. Swimbladder inflation of 12 dah larvae grown in 30‰ was 2.7 and 1.8 times greater than at salinities of 10‰ and 45‰, respectively.

The number of snapper larvae with food in their gut ranged from 70% to 100% and did not vary significantly between salinity treatments for any time of sampling ($P > 0.05$).

Table 3.1. Final percent survival, total length, wet weight, dry weight, incidence of urinary calculi and swimbladder inflation of snapper *Pagrus auratus* larvae grown in different salinities from 3 to 21 dah* (Experiment 1)

Salinity (‰)	Survival (%)	Total length (mm)	Wet weight (mg)	Dry weight (mg)	Urinary calculi (%)	Inflated swimbladders (%)
10	1.3±0.1[a]	6.8±0.1[a]	_____	_____	0[a]	52.2±16.1[a]
15	6.6±2.1[ab]	7.1±0.2[a]	3.0±0.3[a]	0.5±0.1[a]	0[a]	83.3±6.7[a]
20	33.4±10.8[b]	7.1±0.2[a]	3.2±0.2[a]	0.5±0.04[a]	0[a]	76.7±8.8[a]
25	29.2±2.8[b]	6.8±0.1[a]	3.0±0.1[a]	0.5±0.02[a]	10.0±10.0[a]	76.7±23.3[a]
30	18.0±6.6[b]	6.9±03[a]	3.3±0.1[a]	0.6±0.03[a]	80.0±5.8[b]	76.7±3.3[a]
35	19.7±4.3[b]	6.9±0.2[a]	3.0±0.3[a]	0.5±0.1[a]	96.7±3.3[c]	86.7±6.7[a]
45	3.2±1.7[a]	6.0±0.3[b]	_____	_____	100.0±0[c]	68.5±7.1[a]

* Data are means ± standard errors for four (10, 15, 25, 30, 35, 45‰) and three (20‰) replicate tanks.
Within columns, means with a common superscript do not differ ($P>0.05$).
Insufficient numbers of larvae remained in replicate tanks of 10‰ and 45‰ for estimate of wet and dry weights.
All larvae died 48 h after transfer from 35‰ to 5‰.

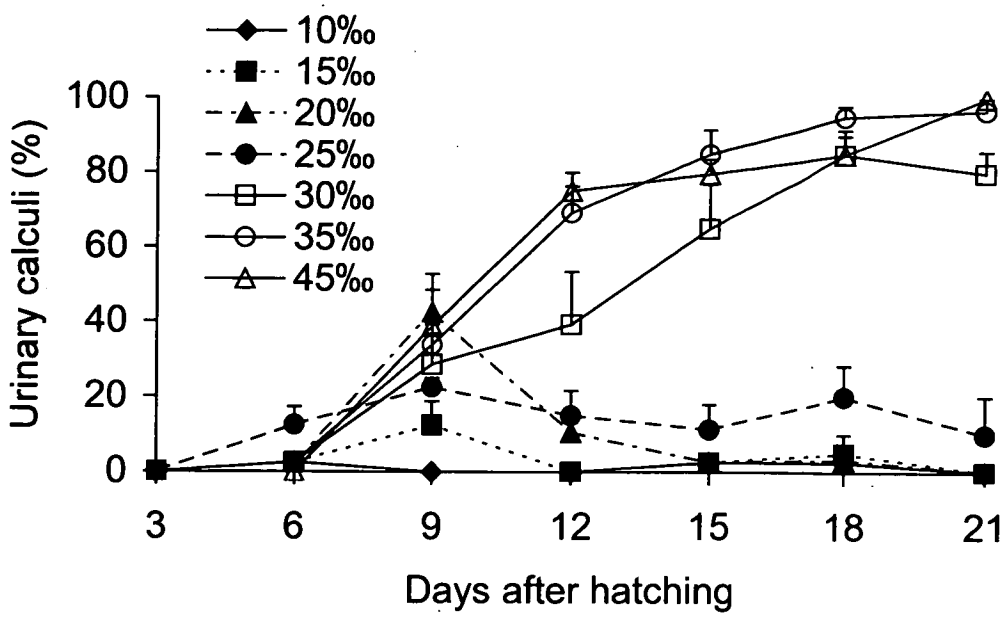

Fig. 3.1. Mean number of snapper, *Pagrus auratus* larvae with urinary calculi grown from 3 to 21 dah in different salinities at 21°C. Data are means for n=4 tanks, except for day 21 which are n=3 tanks. Experiment 1.

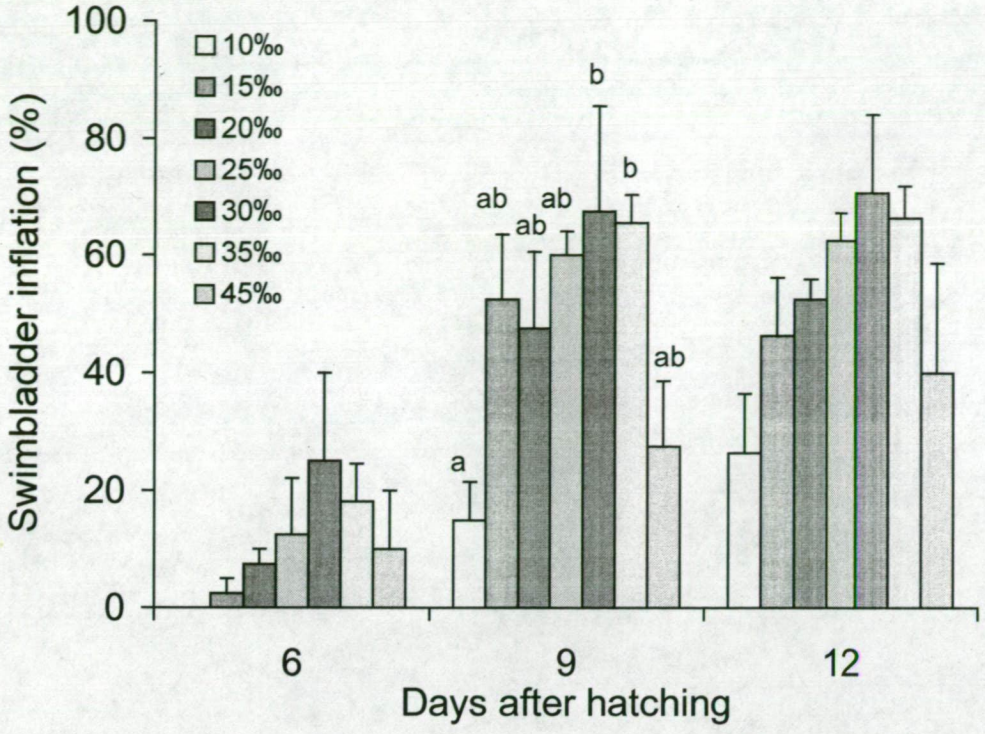

Fig. 3.2. Mean number of snapper, *Pagrus auratus*, larvae with inflated swimbladders grown from 3 dah in different salinities. Data are means ± S.E. for n=4 tanks. At each sampling time, bars with a common superscript do not differ ($P > 0.05$). Experiment 1.

3.4.2. Effect of temperature from 3-21 dah

Temperature had a significant effect ($P < 0.05$) on survival and growth of snapper larvae. All larvae transferred from 21°C to 30°C, 33°C, and 27°C died after 3 and 9 days, respectively. Survival of larvae at 21 dah did not differ significantly when they were grown at 15°C, 18°C, 21°C and 24°C ($P > 0.05$) (Table 3.2), however power of the experiment was low (0.24). At 21 dah, total length, wet weight and dry weight of larvae increased as temperature was increased from 15°C to 24°C (Table 3.2). Temperature had a significant effect ($P < 0.05$) on incidence of urinary calculi. The incidence of calculi occurred earlier and in a greater number of larvae when temperature was increased (Fig. 3.3). The number of larvae with inflated swimbladders was also significantly affected ($P < 0.05$) by temperature (Table 3.2). Larvae grown at 18°C, 21°C and 24°C inflated their swimbladders at high and similar rates. However, larvae grown in 15°C and 27°C inflated their swimbladders at significantly slower rates than those grown in 18°C to 24°C (Fig. 3.4).

The number of snapper larvae with food in their gut ranged from 50% to 100% and did not vary significantly between 15°C to 27°C treatments for any time of sampling ($P > 0.05$).

Table 3.2. Final percent survival, total length, wet weight, dry weight, incidence of urinary calculi and swimbladder inflation of snapper_*Pagrus auratus* larvae grown in different temperatures from 3 to 21dah* (Experiment 2)

Temperature (°C)	Survival (%)	Total length (mm)	Wet weight (mg)	Dry weight (mg)	Urinary calculi (%)	Inflated swimbladders (%)
15	3.8±1.1[a]	5.2±0.04[a]	0.8±0.1[a]	0.1±0.01[a]	7.0±3.5[a]	25.2±4.1[a]
18	16.9±8.3[a]	6.1±0.2[b]	1.5±0.2[ab]	0.2±0.05[ab]	61.1±20.0[b]	65.2±18.0[b]
21	4.6±1.0[a]	6.8±0.3[b]	2.3±0.4[b]	0.4±0.1[b]	83.3±6.7[b]	86.7±8.8[b]
24	4.2±1.5[a]	8.1±0.1[c]	4.8±0.2[c]	0.8±0.03[c]	73.3±6.7[b]	78.9±1.1[b]

* Data are means ± standard errors for three (15°C, 18°C and 21°C) and two (24°C) replicate tanks.

Within columns, means with a common superscript do not differ ($P \geq 0.05$).

All larvae transferred from 21°C to 33°C, 30°C, and 27°C died within 3 and 9 days, respectively.

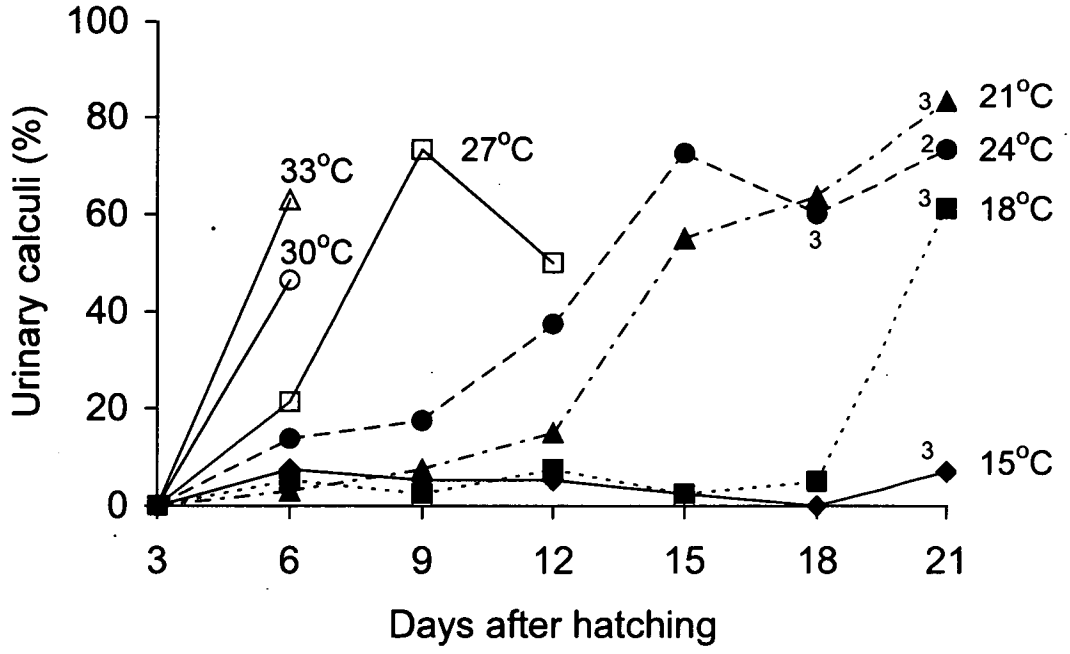

Fig. 3.3. Mean number of snapper, *Pagrus auratus*, larvae with urinary calculi grown from 3 to 21 dah in different temperatures. Data are means for n=4 tanks unless otherwise shown. Experiment 2.

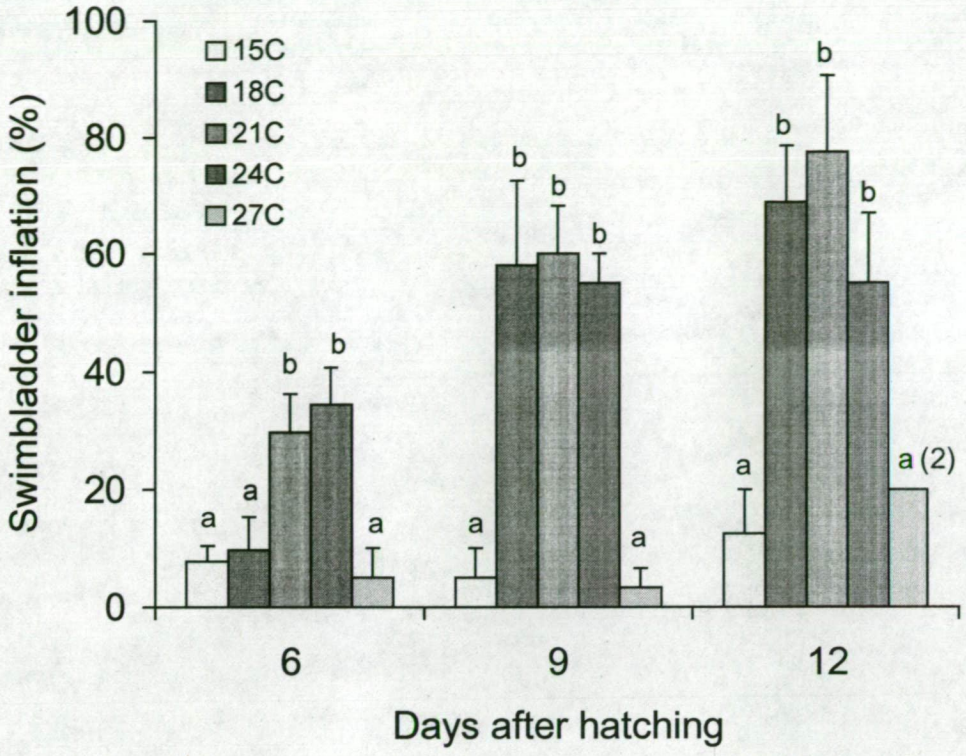

Fig. 3.4. Mean number of snapper, *Pagrus auratus*, larvae with inflated swimbladders grown from 3 dah in different temperatures. Data are means ± S.E. for n=4 tanks unless otherwise shown. At each sampling time, bars with a common superscript do not differ ($P > 0.05$). Experiment 2.

3.4.3. Effect of interaction of salinity and temperature from 3-24 dah.

Neither salinity nor temperature had a significant effect ($P > 0.05$) on survival of snapper larvae (Tables 3.3 and 3.4) however, power of the experiment was low (0.1) due to high variability within treatments. Salinity did not affect final total length, final wet weight and final dry weight ($P > 0.05$), however temperature had a significant effect ($P < 0.05$) on each of these parameters, but there was no salinity x temperature interaction (Tables 3.3 and 3.4). Total length, wet weight and dry weight of larvae increased as temperature was increased (Table 3.3, Fig. 3.5). Larvae in all treatments inflated their swimbladders at similar rates which was not affected significantly ($P > 0.05$) by salinity or temperature (Tables 3.3 and 3.4). The effects of salinity and temperature on the incidence of urinary calculi in larvae were significant ($P < 0.05$) and there was a significant salinity x temperature interaction ($P < 0.05$) (Table 3.4). Very few larvae grown at 20‰ developed urinary calculi during the 21 day experiment and a maximum of 5% of larvae with calculi was achieved at this salinity and 24°C at 9 dah. However, significant numbers of larvae grown at 35‰ developed calculi and the incidence increased as the temperature was increased. Nearly all larvae grown at 35‰ and 24°C had calculi after 9 days, whereas larvae grown at the same salinity and 21°C and 18°C did not achieve 100% incidence of calculi until day 12 and 21 dah, respectively. At day 21 of the experiment (24 dah), there was a marked reduction in the number of calculi present in larvae grown at 35‰ and 24°C (Table 3.3), which were the largest larvae. The number of snapper larvae with food in their gut ranged for 70% to 100% and did not vary between treatment combinations of salinity and temperature for any time of sampling ($P > 0.05$).

Table 3.3. Final percent survival, total length, wet weight, dry weight, incidence of urinary calculi and swimbladder inflation of snapper *Pagrus auratus* larvae grown in different combinations of salinity and temperature from 3 to 24 dah* (Experiment 3)

Salinity (‰)	Temperature (°C)	Survival (%)	Total length (mm)	Wet weight (mg)	Dry weight (mg)	Urinary calculi (%)	Inflated swimbladders (%)
20	18	1.4±2.8 (2)	7.4±0.4 (2)	2.8±2.8 (2)	0.5±0.6 (2)	0 (2)	70.0±19.7 (2)
20	21	3.4±1.8 (5)	9.1±0.2 (5)	7.5±2.3 (3)	1.2±0.5 (3)	0 (5)	73.4±12.5 (5)
20	24	7.8±2.8 (2)	11.4±0.4 (2)	14.9±2.8 (2)	2.6±0.6 (2)	0 (2)	60.0±19.7 (2)
35	18	1.6±2.8 (2)	7.2±0.4 (2)	2.2±4.0 (2)	0.4±0.8 (2)	90.0±6.6 (2)	35.0±19.7 (2)
35	21	4.0±1.8 (5)	9.3±0.2 (5)	6.3±2.3 (3)	1.1±0.5 (3)	98.0±4.2 (5)	50.0±12.5 (5)
35	24	5.1±2.3 (3)	11.1±0.3 (3)	17.6±2.8 (2)	3.1±0.6 (2)	13.3±5.4 (3)	46.7±16.1 (3)

* Data are means ± standard errors. (n) is number of replicate tanks.

Table 3.4. Summary of analysis of variance for effects of combinations of two salinity (20‰ and 35‰) and three temperature (18°C, 21°C and 24°C) levels on *Pagrus auratus* larvae grown from 3 to 24 dah (Experiment 3)

Performance Index	Salinity (‰)	Temperature (°C)	salinity x temperature interaction
Survival	ns[1]	ns	ns
Total length	ns	$P < 0.001$	ns
Wet weight	ns	$P < 0.01$	ns
Dry weight	ns	$P < 0.05$	ns
Urinary calculi	$P < 0.001$	*$P < 0.001$*	$P < 0.001$
Swimbladder inflation	ns	ns	ns

[1]ns = not significant ($P > 0.05$)

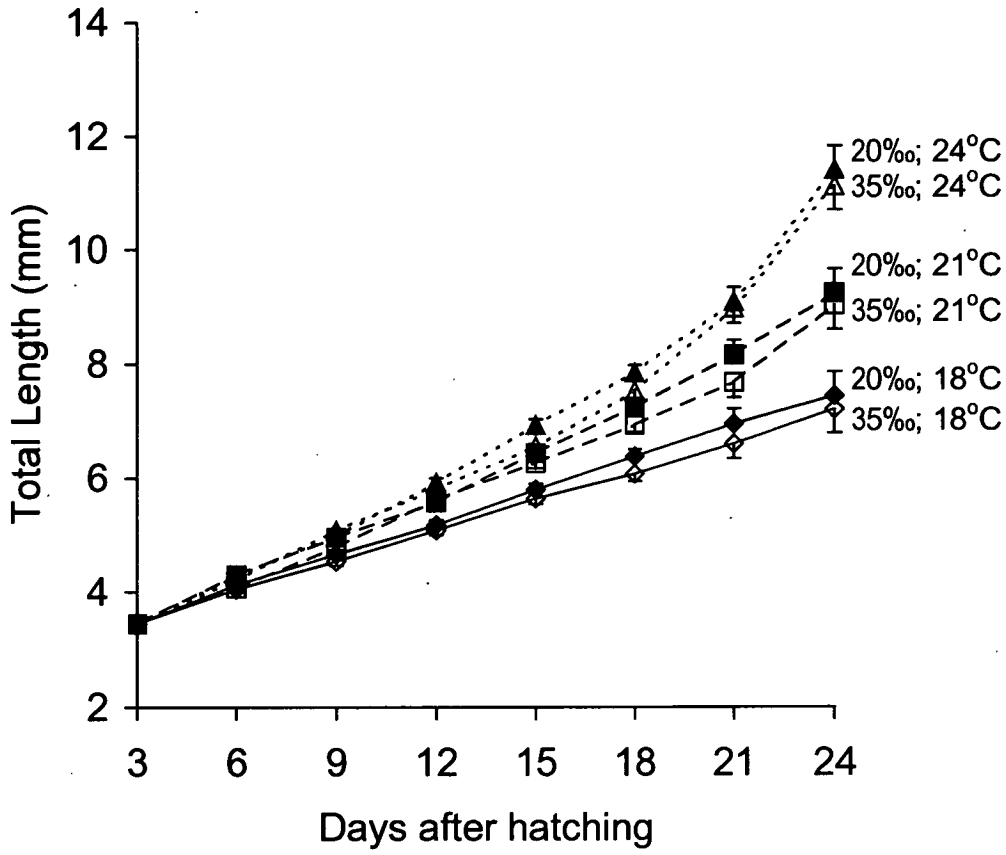

Fig. 3.5. Mean total length of snapper, *Pagrus auratus*, larvae grown from 3 to 24 dah in combinations of two salinity (20‰ and 35‰) and three temperature (18°C, 21°C and 24°C) levels. Data are mean ± S.E. for n=5, 4, 3, and 2 tanks for 6, 9-12, 15-18 and 21-24 dah, respectively.

3.5. Discussion

Salinity and temperature clearly influenced the survival, growth and development of snapper larvae, and in general there was no interaction between combinations of salinity (20‰ and 35‰) and temperature (18°C, 21°C and 24°C), respectively. The effect of temperature within the tested range however, was greater than that of salinity. This finding is consistent with those of other studies where the interactive effects of temperature and salinity on embryonic and larval survival was investigated for sparids such as silver sea bream, *Sparus sarba*, (Mihelakakis and Kitajima, 1994) and red sea bream, *Pagrus major*, (Mihelakakis and Yoshimatsu, 1998). In most cases the effect of temperature far outweighed the effect of salinity (Rombough, 1996).

3.5.1. Effects of salinity on growth and survival

Snapper larvae survived for 18 days (to 21 dah) in all salinities from 10‰ to 45‰ (temperature 21°C) but all 3 dah snapper larvae died within 48 h of transfer from 35‰ to 5‰ (Experiment 1). Survival was best however, at 20‰ to 35‰ and was approximately nine-fold and thirty-fold greater than at salinities of 45‰ and 10‰, respectively. Similarly, Mihelakakis and Yoshimatsu (1998) showed that eggs of red sea bream, a proposed sub-species of *P. auratus* (Tabata and Taniguchi, 2000) did not hatch at 6‰ but viable larvae hatched at the lower and upper salinities of 10-11‰ and 49-50‰, respectively. However, the optimal salinity range for incubation of red sea bream eggs, in terms of larvae that hatched with normal morphology, was 18‰ to 36‰. Optimal salinity for incubation of the closely related silver sea bream, *Sparus sarba*, eggs was also very similar; from 24‰ to 38‰ (Mihelakakis and Kitajima, 1994).

Growth of snapper larvae in terms of total length, wet weight and dry weight, was also the same in salinities from 10‰ to 35‰, but was reduced at the high salinity of 45‰. This

has implications for reduced productivity of snapper in hatcheries located on the Spencers Gulf in South Australia. Ambient salinity of seawater can regularly reach 45‰ due to limited tidal exchange and high evaporation rates (S. Clark, SARDI, pers. comm.). Similarly, Tandler et al. (1995), in a study on another temperate sparid, gilthead sea bream, *Sparus aurata*, showed that survival and wet and dry weights of 32 dah larvae were greatest when salinity was reduced from 40‰ to 25‰ (lower salinities were not tested). Growth of mullet, *Mugil cephalus*, was also better at 20‰ than at 30‰ (De Silva and Perera, 1976).

The reduced growth and survival of snapper larvae at 45‰ is most likely associated with the energy cost of osmoregulation. Metabolic demand of larvae increases in hyperosmotic salinities as they attempt to maintain homeostasis of body fluids. Excess ions, accumulated by drinking seawater to replace osmotic water loss must be actively eliminated (needing energy) to stabilise extracellular fluid concentration (Alderdice, 1988). Therefore, compared with larvae in salinities which are more isoosmotic to body fluids, larvae in hyperosmotic salinities need to divert more energy into metabolism rather than growth. In many marine larvae, including red sea bream (Yamashita, 1978; Hwang and Hirano, 1985), osmoregulation is mostly conducted through chloride cells which develop in the integument of the yolk sac of embryos, on the skin of the epithelium immediately after hatching, and later on the gills (Alderdice, 1988). It is not known, but probable given the extremely close genetic relationship between red sea bream and snapper, that snapper larvae also have chloride cells for osmoregulation. This however, remains to be investigated.

3.5.2. Effects of temperature on growth and survival

Snapper larvae survived to 21 dah at 15°C to 24 °C (salinity 35‰) but all larvae died within 3 to 9 days after transfer (3 dah) from 21°C to 27°C, 30°C and 33°C (Experiment 2), indicating that early stage snapper larvae have a relatively narrow window of temperature tolerance. Snapper spawn naturally in spring and summer in New Zealand (Pankhurst et al., 1991) and Australia (Battaglene, 1995; Fielder, unpublished data) when water temperatures are between 17-22°C and 16-23°C, respectively. The temperature window for early larval snapper survival and development was therefore very similar to the ambient spawning conditions. Similarly, red sea bream eggs hatched from 15°C to 31.1°C (Apostopoulos, 1976) but the optimal hatching temperature range, in terms of number of normal hatched larvae, was 15°C to 22°C (Mihelakakis and Yoshimatsu, 1998). Mihelakakis and Kitajima (1994) also found the optimal temperature range for incubation of silver sea bream eggs was 18°C to 22°C, respectively. Interestingly, these optimal temperature ranges coincide with the natural spawning temperature of each species (Mihelakakis and Yoshimatsu, 1998). In contrast to our findings for pre-metamorphosis snapper larvae, Battaglene and Talbot (1992) showed that 38 dah, metamorphosed snapper tolerated high temperatures up to 31.5°C and did not display any signs of distress, thus confirming that, in general, temperature changes have a more profound effect during early life, and embryos and larvae tend to be more stenothermal than juveniles and adults (Rombough, 1988).

Temperature affects virtually all aspects of reproduction in fish (van Der Kraak and Pankhurst, 1996) and can influence many other aspects of early larval development, such as size at hatching, efficiency of yolk utilisation, growth, feeding rate, time to metamorphosis, behaviour and swimming speed, digestion and gut evacuation rates, and metabolic demand (Blaxter, 1988; Rombough, 1996). In our study, 21 dah snapper larvae

grown at 24°C (8.1 mm TL; 4.8 mg wet weight) were 1.6-fold longer and had 6-fold and 8-fold greater mean wet weights and mean dry weights, respectively than larvae grown at 15°C (5.2 mm TL; 0.8 mg wet weight). The greater increment in the dry weight difference between larvae at 15°C and 24°C most likely reflects the difference in larval development, especially the ossified body parts. Metamorphosis of snapper larvae is completed when larvae are about 8.6 mm in length (Battaglene, 1992) when internal organs (Foscarini, 1988), vertebral column and caudal skeleton (Matsuoka, 1982) and fins (Fukuhara, 1976; cited in Foscarini, 1988; Pankhurst et al., 1991) have developed.

Similar positive relationships between increasing temperature and growth rate of marine fish larvae within the tolerated temperature range over an extended period of time have been demonstrated for gilthead sea bream (Person Le-Ruyet and Verillaud, 1980; Tandler et al., 1989), greenback flounder, *Rhombosolea tapirina*, (Hart et al., 1996), and white flounder, *Pseudopleuronectes americanus*, (Laurence, 1975).

3.5.3. Effects of salinity and temperature on swimbladder inflation

Successful swimbladder inflation is an essential developmental milestone of cultured physoclistous fish larvae (Battaglene, 1995). Failure to inflate functional swimbladders represents a major problem for intensive larval rearing of many fish species (Tandler et al., 1995) and can cause reduced larval growth (Battaglene and Talbot, 1990, 1992), spinal deformities (Takashima, 1978; Chatain, 1994; Kitajima et al., 1994, Trotter et al., 2001) and high, stress-related mortality (Spectorova and Doroshev, 1976; Chatain, 1986, 1987; Chapman et al., 1988; Chatain and Dewavrin, 1989). More is known about the effect of salinity on swimbladder inflation than that of temperature (Battaglene, 1995).

In our study, swimbladder inflation of snapper larvae was strongly influenced by both salinity and temperature. Final swimbladder inflation of larvae grown in salinities between

10‰ and 45‰ did not differ statistically (Experiment 1), however, the initial rate of swimbladder inflation was much slower in larvae grown in 10‰ and 45‰ than in larvae grown at 15‰ to 35‰, which had high rates of swimbladder inflation by 9 dah. At 12 dah, larvae in salinity treatments from 10-45‰ had the same rate of swimbladder inflation but power of the experiment (Searcy-Bernal, 1994) was relatively low (0.4) as the high variability within treatments meant that replication was inadequate. It would therefore be imprudent to accept the null hypothesis that salinity from 10-45‰ did not affect swimbladder inflation of snapper larvae. The mean number of snapper larvae at 12 dah grown in 30‰ with inflated swimbladders was 2.7 and 1.8 times greater than at 10‰ and 45‰, respectively. Survival of 21 dah snapper larvae grown in 10‰ and 45‰ was only 4% and 9.5% of larval survival at 20‰, respectively. Therefore it is likely that larvae which did not inflate their swimbladders died before the end of the experiment (Battaglene and Talbot, 1990). Although multiple comparisons of means did not clearly identify treatment differences due to insufficient replication, at 12 dah, when swimbladder inflation of snapper larvae (at ~21°C) is completed (Battaglene and Talbot, 1992; Fielder et al., 2002, Chapter 2), only 26.4% and 40% of larvae reared in 10‰ and 45‰, respectively had inflated swimbladders compared with 50-71% for larvae reared in 20-35‰. Data from Experiment 3 confirmed that swimbladder inflation of snapper larvae is not affected at salinities from 20‰ to 35‰.

The effect of salinity on swimbladder inflation is species-specific. As in our study, rate of swimbladder inflation of Australian bass, *Macquaria novemaculeata*, larvae was high in a range of salinities from 15‰ to 35‰ but was low at 10‰ (Battaglene and Talbot, 1990; Battaglene, 1995). Low salinities may mean that larvae are not buoyant enough to swim to the surface to gulp air at initial swimbladder inflation (Hadley et al., 1987; Howell et al., 1998). Tandler et al. (1995) also showed that high salinity caused a reduction in

swimbladder inflation of gilthead sea bream but when salinity was reduced from 40‰ to 25‰ and larvae were less than 13 dah, swimbladder inflation improved by 30%. Stress-induced hypertrophy of, and failure to, inflate swimbladders in European sea bass, *Dicentrarchus labrax* larvae at 36‰ was ameliorated when salinity was reduced (Johnson and Katavic, 1984). It is therefore possible that larval stress at high salinity (45‰) was responsible for poor swimbladder inflation in snapper larvae.

Little information exists regarding the effects of varying temperature on swimbladder inflation (Battaglene, 1995). Gibson and Johnson (1995) showed that organogenesis of turbot, *Scophthalmus maximus*, larvae varied with temperature, including swimbladders, which at 12°C were inflated while some yolk reserves remained, but at 16°C swimbladders were inflated after the yolk was fully depleted. Striped bass, *Morone saxatilis*, larvae inflated swimbladders more successfully at 16°C than at 19°C (Hadley et al., 1987). In contrast, in our study swimbladder inflation of snapper larvae at 15°C and 27°C was approximately 3-fold lower than inflation at 18-24°C. All larvae at 27°C died by 12 dah suggesting that this temperature was too high to support a range of developmental and metabolic processes other than swimbladder inflation. On the other hand, survival of larvae to 21 dah at 15°C was the same as that of higher temperature treatments. Snapper larvae usually inflate their swimbladders when they are approximately 3.5-4.5 mm TL (Battaglene and Talbot, 1992; Fielder et al., 2002, Chapter 2). Larvae grew (albeit slowly) to a mean of 5.2 mm TL at 15°C, suggesting that the opportunity for swimbladder inflation was missed. It is possible that swimming vigour of snapper larvae at this temperature was reduced, which prevented larvae from reaching the water surface to gulp air.

3.5.4. Effects of salinity and temperature on incidence of urinary calculi

Salinity and temperature both influenced the presence of calculi in the urinary bladder and there was a significant interaction in combination of 20-35‰ and 18-24°C. Urinary calculi did not develop in larvae at salinity of 25‰ or lower but after 18 d (21 dah) at 30-45‰ most larvae had developed urinary calculi. The presence of urinary calculi was not associated with swimbladder abnormalities and there was no relationship between urinary calculi and larval survival. Urinary calculi also developed earlier (more rapidly) and in greater numbers of larvae as temperature was increased. Although all larvae in the high temperature treatments of 27-33°C died after 3-9 days of imposing the treatments, it is likely that physiological dysfunction in this temperature range caused larval death, rather than the presence of urinary calculi. The large number of surviving, growing larvae with calculi at 21 dah in 15-24°C supports this. It appears that the presence of urinary calculi changes with ontogeny of snapper larvae. In Experiment 3, all larvae grown at 35‰ and 24°C had developed urinary calculi at 21 dah (9.0 mm TL) but at 24 dah when larvae were 11.1 mm TL (17.6 mg wet weight) only 13% of larvae had urinary calculi. Snapper larvae in this treatment were more developed and 3-fold heavier than larvae grown in 35‰ and 21°C. This indicates therefore that a critical minimum larval size may be necessary to allow the expulsion of calculi from the urinary bladder, or alternatively the physiology of the larvae, which allows excretion of the calculi changes with development.

Urinary calculi have been observed in hatchery-reared sparids, including sharpsnout seabream, *Diplodus puntazzo*, gilthead sea bream, and snapper (Modica et al., 1993; Battaglene, 1995; Favaloro and Mazzola, 2000) as well as European sea bass (Boglione et al., 1993; Menu et al., 1998), Japanese flounder, *Paralichthys olivaceus*, (Sakai et al., 1996) and brown-marbled grouper, *Epinephelus fuscoguttatus*, (Sivaloganathan et al., 1996). Similar to our study, the presence of urinary calculi in larvae and juveniles of

sharpsnout seabream (Favaloro and Mazzola, 2000), snapper (Battaglene, 1995) and Japanese flounder (Sakai et al., 1996) was not associated with larval mortality and did not influence the growth performance of fish. Battaglene (1995) found that in snapper, the presence of urinary calculi persisted at least until weaning and then suggested they became difficult to see using light microscopy due to the formation of scales and pigmentation of larvae. Our results suggest that this author may have also failed to see the calculi in snapper because they had been expelled or dissolved when larvae reached a critical size. In contrast, Modica et al. (1993) suggested that presence of urinary calculi was a pathological problem and caused death of 55 dah gilthead sea bream.

3.5.5. General

Salinity and temperature can affect feeding efficiency of larvae by influencing processes such as metabolism, oxygen consumption, behaviour and swimming speed, and gut evacuation time (Blaxter, 1988; Ronzani Cerqueira, 1991). In addition, availability of live feeds (rotifers) to larvae can be influenced strongly by transfer shock following rapid changes in salinity and temperature (Fielder et al., 2000). We attempted to avoid problems with transfer shock of rotifers when transferred from culture conditions to treatment tanks (with different environmental conditions) by rearing rotifers at salinities and temperatures close to treatment conditions. Snapper larvae commenced and maintained feeding at similar rates in all combinations of salinity from 10‰-45‰ and temperature from 15°C-27°C.

Slight differences in survival and development of larvae occurred in control salinity (35‰) and temperature (21°C) treatments between experiments, despite attempts to maintain the same rearing conditions. Initial stocking densities of snapper larvae in experiments ranged from 6-15 larvae l^{-1}, which is low compared with other intensively

reared sparids; 12-72 larvae l^{-1} for red sea bream (Fukusho, 1989) and 100 larvae l-1 for gilthead sea bream, (Tandler et al., 1989; Chatain and Ounais-Guschemann, 1990). In addition to low larvae stocking density, rotifers were provided at high densities (Parra and Yúfera, 2000) to avoid density effects on larval performance. A possible explanation for our differences relates to larvae for each experiment being different progeny. Hadley (1987) suggested that variations in development of striped bass were due to variation between progenies. The quality of eggs and larvae can be influenced by broodstock nutrition and genetics which in turn can influence larval survival and quality (Furuita et al., 2000; Bromage, 2001). In addition, the environmental conditions including salinity and temperature to which broodstock fish are exposed prior to spawning can influence the tolerance and performance of larvae (Bromage, 2001). Although we attempted to maintain broodstock snapper under similar conditions, salinity and temperature varied up to approximately 5‰ and 2°C, respectively between spawning events. It is possible that these slight differences influenced the performance of the different progenies (groups) in our experiments. Alternatively, variation between experimental results may have been caused by rearing larvae in relatively small tanks, which are not necessarily ideal for optimal survival and growth of fish larvae.

Salinity and temperature do not have independent effects on development of embryos and larvae and, ideally, should be considered together when determining optimal conditions for larval rearing of fish (Howell et al., 1998). Short-term effects of salinity and temperature on performance of eggs, larvae and juvenile marine fish have been observed in many studies (e.g. Kinne, 1963; Alderdice and Forrester, 1968, 1971; Holliday, 1969; Santerre, 1976; Freddi et al., 1981; Rombough, 1996), however, few studies have investigated the effects of long-term exposure (Tandler et al., 1995). The latter is essential to determine optimum rearing conditions for larvae (Hart et al., 1996; Tandler et al., 1995).

In our study, salinity (20‰ and 35‰) and temperature (18°C to 24°C) were independent of each other in their effect on larval survival, growth and swimbladder inflation but were not independent of each other in their effect on the presence of urinary calculi. Based on our results in terms of larval performance (growth), development and survival we conclude that the optimal conditions for larval rearing of snapper from first-feeding (3 dah) to pre-metamorphosis (24 dah) are combinations of salinity from 20‰ to 35‰ and a temperature of 24°C.

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CHAPTER 4

Combined effects of photoperiod, salinity and temperature on growth and survival of snapper *Pagrus auratus* larvae in commercial-scale tanks

4. Combined effects of photoperiod, salinity and temperature on growth and survival of snapper *Pagrus auratus* larvae in commercial-scale tanks

4.1. Summary

The performance of Australian snapper, *Pagrus auratus* larvae from 4-33 days after hatch (dah) under two environmental rearing regimes was evaluated in 2000-l commercial-scale larval rearing tanks. The treatments were: (1) a "new" regime of salinity (20-35‰), temperature (24°C) and photoperiod (12L:12D to swimbladder inflation, then 18L:06D), and (2) a previous "best-practice" regime of salinity (35‰), temperature (21°C) and photoperiod (14L:10D). The final total length (TL), and wet and dry weights of larvae grown in the "new" regime were greater (15.6 mm; 42.4 mg wet weight; 7.3 mg dry weight) than those of larvae in the previous "best-practice" (11.1 mm; 12.9 mg wet weight; 2.1 mg dry weight). By 33 dah, larvae in the "new" regime were fully-weaned from live feeds to a formulated pellet diet and were suitable for transfer from the hatchery to a nursery facility. In contrast, larvae in the previous "best-practice" regime were not weaned onto a pellet diet and still required live feeds. Neither survival (10-20%) nor swimbladder inflation (70% by 13 dah) were affected by rearing regime. The incidence of urinary calculi at 7 dah was greatest initially in the "new" regime, however by 19 dah, when larvae were 8.0 ± 0.28 mm TL, very few larvae in this treatment had urinary calculi. In contrast, many larvae in the previous "best-practice" regime had developed urinary calculi and this continued until the end of the experiment. The incidence of urinary calculi was not associated with larval mortality. Extrapolation of the snapper larval growth curves for the previous "best-practice" larval rearing regime predicts that a further 15-18 days, or approximately 1.5-times longer, will be required until these larvae attain the same size and development of larvae reared in the "new" regime. Approximately eleven hatchery cycles

per year are possible when larvae are reared under the "new" regime compared with seven hatchery cycles per year for the previous "best-practice" regime.

4.2. Introduction

Knowledge of environmental requirements is important when siting fish hatcheries and when designing and managing facilities (Howell et al., 1998). In many cases, environmental requirements for aquaculture species are assumed to be suitable for others however this is often not the case and optimal environmental conditions may change during ontogeny for a single species (Blaxter, 1969).

Three of the most important abiotic parameters which influence the performance of fish larvae are salinity, temperature and photoperiod (e.g. Kinne, 1963; Battaglene, 1995; Rombough, 1996; Hart et al., 1996; van der Kraak and Pankhurst, 1996; Howell et al., 1998; Boeuf and Le Bail, 1999). Salinity can affect growth rate and survival of larvae by influencing energetics associated with osmoregulation (Kinne, 1963; Howell et al., 1998), swimbladder inflation (Battaglene and Talbot, 1990, 1993) and the formation of abnormal calculi in the urinary bladder and kidney (Modica et al., 1993; Sakai et al., 1996).

Temperature can affect almost every aspect of fish larval development including survival, growth, feeding, digestion and metabolism (Blaxter, 1988; Rombough, 1996; van der Kraak and Pankhurst, 1996). Most marine fish larvae require light to feed and growth of larvae can be increased by increasing photoperiods (Barahona-Fernandes, 1979; Blaxter, 1980; Duray and Kohno, 1988; Barlow et al., 1995; Hart et al., 1996; Boeuf and Le Bail, 1999). However, the optimal photoperiod for survival is not necessarily the same as that for optimal growth (Barahona-Fernandes, 1979; Ronzani Cerqueira and Chatain, 1991) and may change during ontogeny (Fielder et al., 2002, Chapter 2).

Many studies have investigated the short-term effects of salinity, temperature and photoperiod on marine fish larvae either alone or in combination to determine interactive effects of the parameters (for reviews see: Alderdice, 1988; Blaxter, 1988; Rombough, 1996; Boeuf and Le Bail, 1999). These studies have generally been done in small tanks

from 1-l to 3-l (Perschbacher et al., 1990; Parado-Estepa, 1991; Hart et al., 1996; Bolla and Ottesen, 1998; Specker et al., 1999) or medium size tanks of 100-l to 150-l (Steinarsson and Björnsson, 1999; Fielder et al., 2002), probably for logistical reasons to allow replication of several treatments. On the other hand, few studies have determined the long-term effects of environmental conditions on larval performance (Tandler et al., 1995; Hart et al., 1996) and even fewer studies have been done in large, commercial-scale tanks (e.g. >600-l). This latter evaluation is important to obtain information on potential juvenile fish production and hatchery costs as well as investigating potential difficulties with scaling-up from experimental to commercial hatchery production. Other factors which influence performance of fish larvae, such as light intensity, turbulence and aeration (Chatain and Ounais-Guschemann, 1991; Opstad and Bergh, 1993; Huse, 1994; Battaglene, 1995; Cobcroft et al., 2001), may differ with tank size. The influence of small tank environmental parameters in combination with the salinity, temperature and photoperiod effects may therefore affect fish larval performance to a greater or lesser degree in large-scale, commercial production tanks.

Culture of Australian snapper, *Pagrus auratus*, is increasing in Australia (Battaglene and Fielder, 1997; Fielder et al., 2001; Fielder et al., 2002, Chapter 2) and a priority has been placed by industry on research to increase production of juvenile snapper and to reduce the costs of production. Methods have been developed for reliable spawning of high quality eggs (Cleary, 1997; Fielder et al., 1999) and the optimal salinity, temperature and photoperiod for larval rearing have been identified in experiments which addressed the effects of these parameters in small, 100-l experiment tanks (Fielder et al., 2002, Chapters 2 and 3). These authors recommended a "new" salinity (20-35‰), temperature (24°C) and photoperiod (12L:12D to swimbladder inflation, then 18L:06D) regime because growth of snapper larvae was much greater than that of larvae grown under the previous "best-

practice" regime of salinity (35‰), temperature (21°C) and photoperiod (14L:10D). The "best-practice" regime was based on the ambient conditions during the natural spring spawning season from August to November (Battaglene and Talbot, 1992) and was used by commercial hatchery operators in New South Wales, South Australia and Western Australia. However, the performance of snapper larvae under the "new" regime in commercial-scale tanks has not been evaluated.

The aim of the study was to evaluate the performance of snapper larvae in terms of growth, survival, swimbladder inflation and development, from first-feeding to 33 days after hatch (dah) under the "new" and "best-practice" rearing regimes in commercial-scale tanks.

4.3. Materials and Methods

4.3.1. Source of larvae

Fertilised eggs were obtained after first generation hatchery-reared broodstock spontaneously spawned in a 17,000-l flat-bottomed tank, that was part of an independent, recirculating system operated with an external 700-l egg collection sump, pump and mechanical and biological filters. The tank was held in a temperature and photoperiod controlled room.

Fertilised eggs were transferred to a 750-l flat-bottomed tank filled with sterilised seawater (20 mg l^{-1} sodium hypochlorite for 24 h then neutralised with sodium thiosulphate) for incubation under darkness, salinity and temperature of 34.5‰ and 21 ± 1°C. Approximately 50% of the tank volume was drained daily and replaced with sterilised seawater. The tank had slight aeration (~200 ml min^{-1}) and was siphoned daily to remove unhatched eggs and detritus. Surface films were removed by soaking strips of blotting

paper on the water surface. Larvae remained in the incubation tank for 4 days after hatching (dah).

4.3.2. Experiment tanks

The experiment was done in 2,000-l conical-bottom tanks with black sides and white bottoms, which were contained in an insulated room. Each tank was independent of the others and had individual recirculation of water at 5-15 l min^{-1} through mechanical and biological filters. Water temperature in each tank was controlled by a titanium immersion heater (3 KW, Austin and Cridland, Australia) and surface skimmers were provided in each tank to remove surface films. Fluorescent lighting (Philips White TLA 40W 33QS) was provided overhead at 8.7 μmol s^{-1} m^{-2} and each light was operated by a separate time switch. Each tank was enclosed within a box made from black plastic sheet to prevent reflected light affecting surrounding tanks. Approximately 15% of the water in each tank was exchanged every day with either sterilised seawater or fresh groundwater (0.6‰) to maintain (Treatment 1) or reduce salinity (Treatment 2). Water pH was maintained by adding sodium carbonate as required.

4.3.3. Experimental design

The experimental treatments were:

(a) The previous "best-practice" environmental regime (Treatment 1)

salinity – 35‰

temperature – 21°C

photoperiod – 14L:10D (light:dark)

(b) "New" environmental regime (Treatment 2)

salinity – 25‰ (within the optimal range of 20‰ to 35‰ determined previously; Chapter 3)

temperature – 24°C

photoperiod – 12L:12D until swimbladder inflation, then 18L:06D.

Four days after hatching a randomly selected sample of 60 larvae was collected from the incubation tank to determine total length (3.3 ± 0.11 mm total length [TL], mean ± S.D.). Larvae were then mixed homogeneously by slightly increasing aeration in the incubation tank. The number of larvae in the incubation tank was estimated by randomly selecting five 2-l samples of larvae. The volume in the incubation tank was then reduced from 750 l to 300 l by siphoning through a 200μm mesh screen and five, 10-l buckets of larvae were added to each of six experiment tanks filled with seawater (35.4 ± 0.2‰, 21.8 ± 0.4°C, mean ± S.D., $n = 6$). The initial number of larvae that were stocked into each tank was 60,340 ± 4820 larvae $tank^{-1}$ (30.2 larvae l^{-1}). Three randomly selected replicate tanks were allocated to each treatment.

After stocking, temperature in replicate tanks of Treatment 2 was increased from 21°C to 24°C over 48 h and salinity in these tanks was slowly lowered during the course of the experiment to approximately 25‰ (Table 4.1). When larvae were 13 dah the photoperiod of tanks in Treatment 2 was increased from 12L:12D to 18L:06D.

4.3.4. Larvae feeding and sampling

Snapper larvae in each treatment were fed a succession of rotifers, artemia metanauplii and artificial weaning pellet ("ML powered", Nippai, Japan). Artemia and pellet were introduced in different treatments when larvae reached a predetermined size (Fig. 4.1) but the age at which the diet was changed and the density that live feeds were provided varied

Table 4.1.

Physical parameters for 2000-l experiment tanks used to rear snapper, *Pagrus auratus* larvae from 3 to 33 dah.Target treatments are: Previous "best-practice", Treatment 1 - 35‰, 21°C,14:10 L:D; "New", Treatment 2 - 25‰, 24°C, 12:12 L:D to swimbladder inflation then 18:06 L:D. Data are means ± S.E. (n = 30 days).

Treatment	Replicate tank	Daylength (L:D)	Salinity (‰)	Temperature (°C)	DO_2 (mg l^{-1})	pH	TAN (mg l^{-1})
1	1	14:10 from 3-33 dah	34.4±0.5	20.6±0.1	7.0±0.1	7.8±0.02	0.15±0.02
	2	"	34.4±0.5	20.7±0.1	7.3±0.1	7.8±0.02	0.09±0.02
	3	"	34.1±0.5	20.9±0.1	7.1±0.1	7.8±0.02	0.08±0.02
2	1	12:12 from 3-13 dah 18:06 from 13-33 dah	26.7±1.1	23.7±0.1	6.5±0.1	7.7±0.02	0.08±0.02
	2	"	26.8±1.1	24.0±0.1	6.5±0.1	7.8±0.02	0.08±0.02
	3	"	26.2±1.1	23.7±0.1	6.4±0.1	7.7±0.02	0.01±0.01

between treatments according to size of larvae and the daily food consumption rate (Table 4.2). The density of rotifers and artemia remaining in the tanks, as well as accumulation of uneaten pellet, was monitored during each day and if necessary, adjustments were made to the feeding schedule. Rotifers were maintained between 5-10 rotifers ml^{-1}. Rotifers, *Brachionus plicatilis* (size range 160-260 μm lorica length) and artemia metanauplii were enriched prior to feeding to snapper larvae using methods described by Fielder et al., 2002. Pellet diet was provided continuously from an automatic belt-feeder (AGK, Melbourne, Australia) during light periods.

A randomly selected sample of ten larvae was collected from each tank at approximately 1300 h every 3 days and live larvae were observed under a dissecting microscope fitted with an ocular micrometer to determine presence/absence of inflated swimbladders, food in the gut, urinary calculi and development of tail flexion. Another sample of ten live larvae with swimbladders was measured to determine total length (TL = distance from the tip of the lower jaw to lower tip of the caudal fin).

The wet and dry weights of larvae with swimbladders were measured in three randomly selected groups of ten larvae from each tank every 6 days. Surface water on larvae was removed using blotting paper and larvae were then placed onto a single, pre-weighed glass microscope slide (n = 10 larvae) and larvae weight was determined to the nearest 0.01 mg with an analytical balance ("Analytical Plus", Ohaus Corporation, Switzerland). The slides were then placed into a drying oven at 106°C for 16 h after which time each slide was weighed to the nearest 0.01 mg to estimate final larvae dry weight. The final number of surviving fish was estimated by concentrating harvested fish from each experiment tank into a 100-l tank. The fish were then anaesthetised with 50 mg l^{-1} of ethyl-p amino benzoate (Sigma, Australia) until they lost equilibrium of balance and

Table 4.2.

Feeding regime of snapper, *Pagrus auratus* larvae grown in 2,000-l tanks from 4 to 33 dah under different salinity, temperature and photoperiod conditions.

Days after hatch	Treatment 1 (35‰; 21°C; 14:10 L:D)						Treatment 2 (25‰; 24°C; 12:12, 18:06 L:D)					
	Rotifers[1]		Artemia[2]		ML pellet[3]		Rotifers[1]		Artemia[2]		ML pellet[3]	
	(no. ml^{-1})	(feeds d^{-1})	(no. ml^{-1})	(feeds d^{-1})	weight (g)	size (µm)	(no. ml^{-1})	(feeds d^{-1})	(no. ml^{-1})	(feeds d^{-1})	weight (g)	size (µm)
4 to 15	10	2					10	2				
16	10	2					10	2			5	100
17	10	2					10	2			5	100
18	10	2					10	2			5	100
19	10	2					10	2			5	100
20	10	2					10	2	0.2	2	10	100
21	10	2					10	2	0.2	2	10	100
22	10	2			5	100	10	2	0.2	2	5 , 5	100 , 200
23	10	2			5	100	10	2	0.2	3	5 , 5	100 , 200
24	10	2			10	100	10	2	0.2	4	5 , 5	100 , 200
25	10	2	0.2	2	5 , 5	100 , 200			0.2	6	10 , 10	100 , 200
26	10	2	0.2	3	5 , 5	100 , 200			0.2	8	10 , 10	100 , 200
27	10	2	0.2	3	5 , 5	100 , 200			0.3	6	10 , 10	100 , 200
28	10	2	0.2	3	5 , 5	100 , 200			0.2	3	20	200
29	10	2	0.2	3	5 , 5	100 , 200			0.2	3	25	200
30	10	2	0.2	3	5 , 5	100 , 200			0.2	2	25	200
31	10	2	0.2	4	5 , 5	100 , 200			0.2	1	30	200
32	10	2	0.2	6	5 , 5	100 , 200					35	200
33	10	2	0.2	8	5 , 5	100 , 200					40	200

[1]rotifers were added to maintain the target density. The no. ml^{-1} is maximum rotifer density.

[2]total number fed per day is no ml^{-1} x feeds day^{-1}

[3]total weight fed per day is weight x feeds day^{-1}. Pellet was provided continuously from a belt feeder during light hours .

stopped swimming. The fish were then mixed homogeneously by heavy aeration and five, 2-l samples were selected randomly to determine the mean number of surviving fish.

4.3.5. Daily water measurement

Salinity, temperature, pH and DO_2 were measured daily in all replicates using a water quality meter (Horiba U-10, Horiba Ltd, Japan) (Table 4.1). Total ammonia was measured daily in at least one replicate tank from each treatment with a rapid test kit (E. Merck,

Model 1.08024, Germany) (Table 4.1). Light intensity was measured to the nearest 0.1 $\mu mol\ s^{-1}\ m^{-2}$ with a light meter (LI-COR, model Li-1776, USA).

4.3.6. Statistical analyses

Data were analysed using single factor analysis of variance (ANOVA). Homogeneity of variance was confirmed by Cochran's test (C; Winer et al., 1991) and where necessary, data were transformed (log (x)), to satisfy the assumption of homogeneity of variance. Where significant differences were found with ANOVA means were compared by the Student-Newman-Keuls test (SNK). Statistical analyses were done using Statgraphics Version 5.0 (STSC Inc., USA).

4.4. Results

Combinations of different salinity, temperature and photoperiod had a significant effect ($P < 0.05$) on growth and performance of snapper larvae. Larvae in Treatment 2 (25‰, 24°C, 12:12 then 18:06 L:D) were significantly longer ($P < 0.05$) than those in Treatment 1 (35‰, 21°C, 14:10 L:D) at 16 dah and this trend continued until the end of the experiment at 33 dah when Treatment 2 larvae were 1.4-fold longer than Treatment 1 larvae (Fig. 4.1). The TL increment of snapper larvae was expressed as a polynomial relationship for Treatment 1 ($y = 0.00252x^2 + 0.169x + 2.59$; $R^2 = 0.996$) and Treatment 2 ($y = 0.0077x^2 + 0.121x + 2.70$; $R^2 = 0.992$); where y = TL (mm) and x = dah . Similarly, final wet and dry weights of larvae in Treatment 2 were 3.4-fold heavier (Figs. 4.2 and 4.3) than those of larvae in Treatment 1. Significant differences ($P < 0.05$) in wet and dry weights between treatments were detected 3 days earlier (at 13 dah) than those of TL (Figs. 4.2 and 4.3).

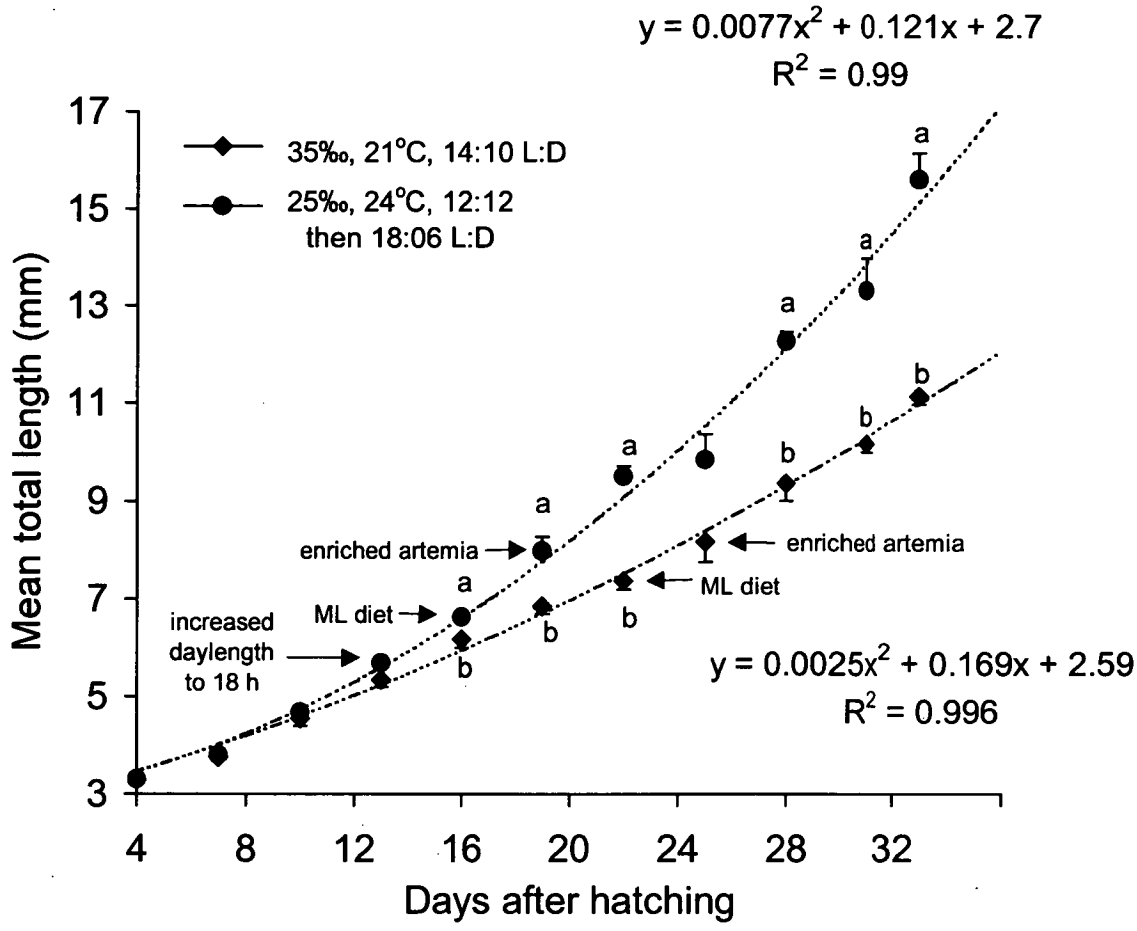

Fig. 4.1. Mean total length of snapper, *Pagrus auratus*, larvae grown in 2,000-l commercial tanks from 4 to 33 dah under different salinity, temperature and photoperiod regimes. Data are means ± S.E. for n=3 tanks. At each sampling time, points with a different superscript are significantly different ($P < 0.05$). The equations for total length increment for each treatment are described, where y = total length (mm) and x = dah.

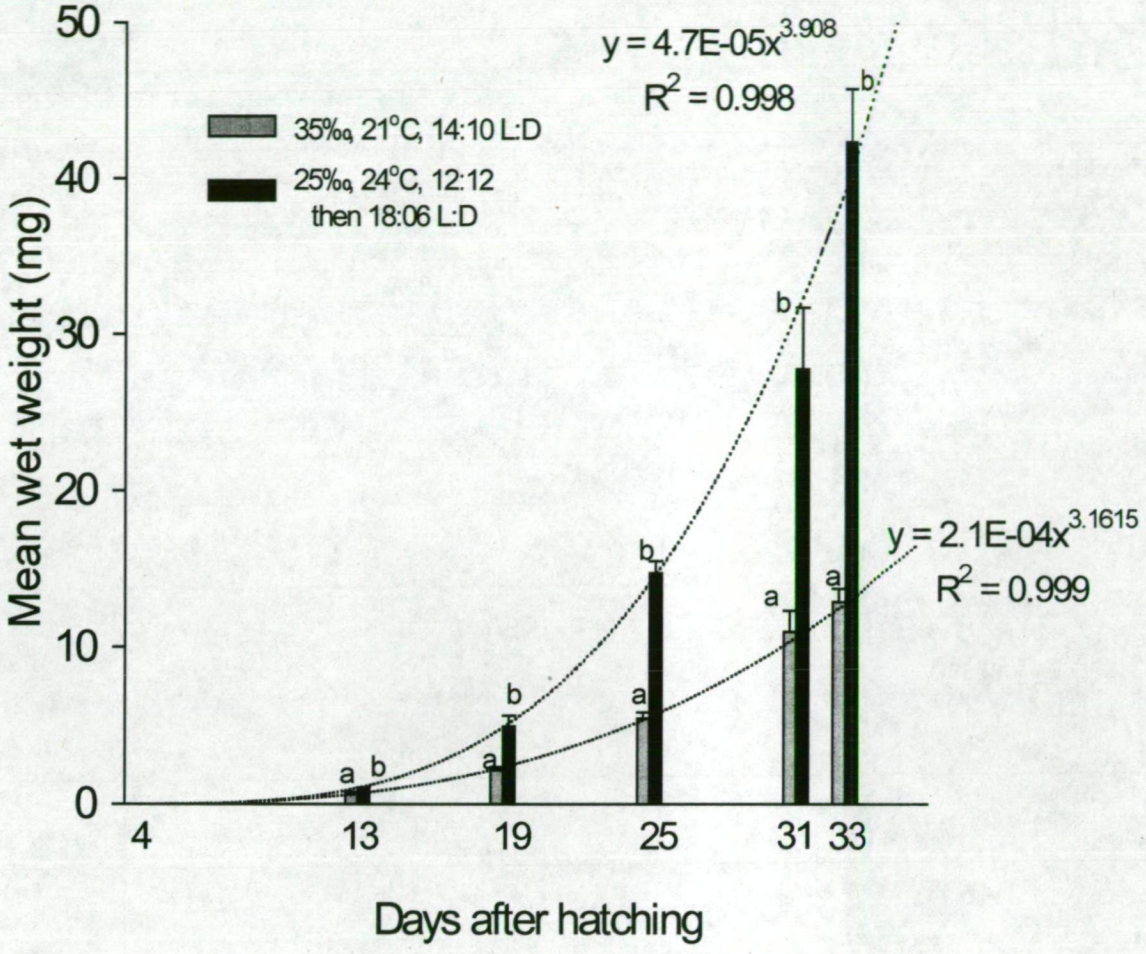

Fig. 4.2. Mean wet weight of snapper, *Pagrus auratus*, larvae grown in 2,000-l commercial tanks from 4 to 33 dah under different salinity, temperature and photoperiod regimes. Data are means ± S.E. for n=3 tanks. At each sampling time, bars with a different superscript are significantly different ($P < 0.05$). The equations for wet weight increment for each treatment are described, where y = wet weight (mg) and x = dah.

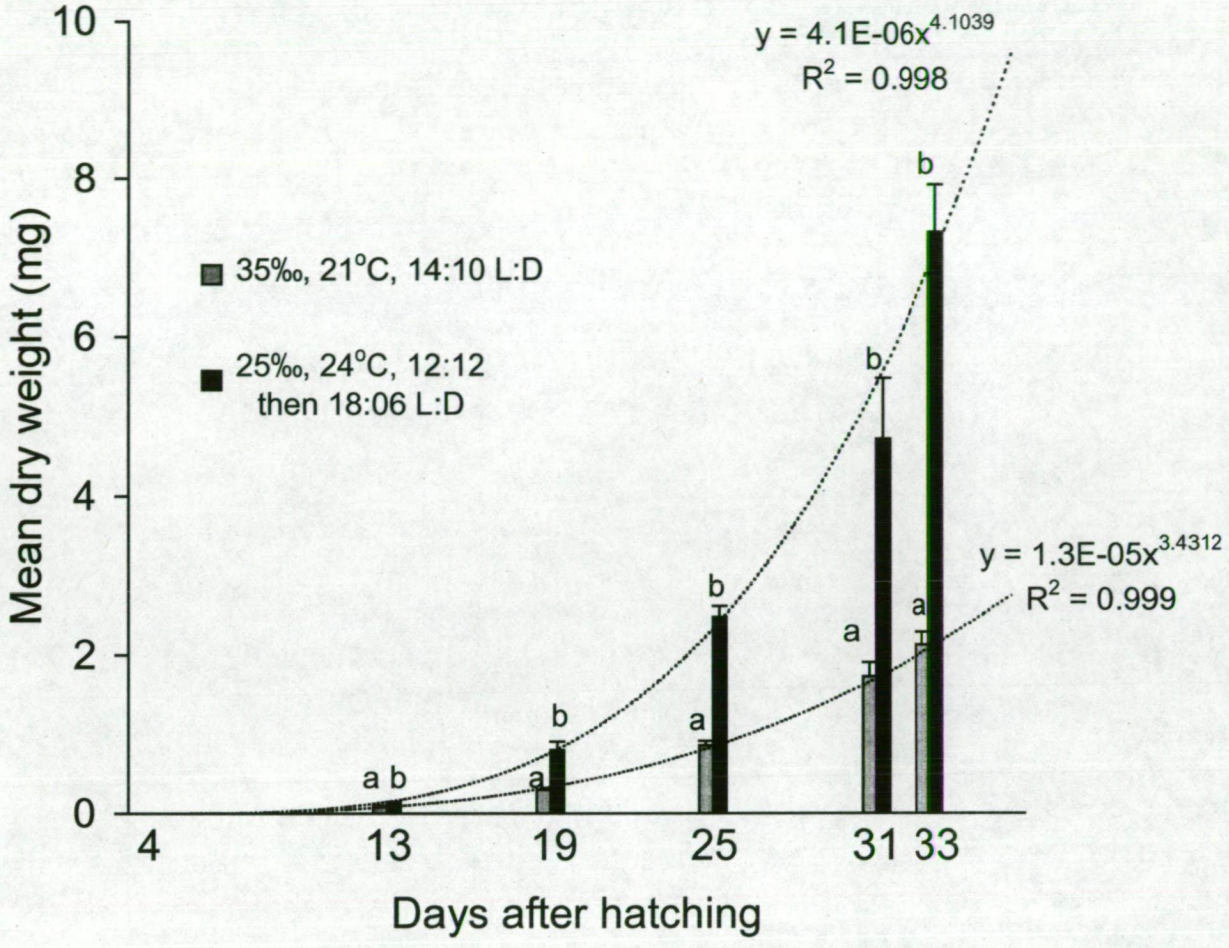

Fig. 4.3. Mean dry weight of snapper, *Pagrus auratus*, larvae grown in 2,000-l commercial tanks from 4 to 33 dah under different salinity, temperature and photoperiod regimes. Data are means ± S.E. for n=3 tanks. At each sampling time, bars with a different superscript are significantly different ($P < 0.05$). The equations for dry weight increment for each treatment are described, where y = dry weight (mg) and x = dah.

Survival of larvae to 33 dah was not significantly different ($P > 0.05$) between treatments (Fig. 4.4), however, power of the experiment to detect treatment differences was low (0.1) (Searcy-Bernal, 1994). Treatments did not affect ($P > 0.05$) swimbladder inflation, which was 70% for both treatments at 13 dah (Fig. 4.5) but did affect development of tail flexion ($P < 0.05$). Nearly all larvae in Treatment 2 had started tail flexion at 16 dah, whereas development of larvae in Treatment 1 was slower and larvae did not attain similar rates of tail flexion until 25 dah (Fig. 4.6).

The incidence of larvae with urinary calculi was significantly affected ($P < 0.05$) by treatment and changed during ontogeny (Fig. 4.7). The incidence of calculi at 7 dah was greatest initially in Treatment 2, but by 19 dah, when larvae were 8.0 ± 0.28 mm TL, very few larvae in this treatment had urinary calculi. In contrast, the number of larvae with urinary calculi in Treatment 1 started off low (< 10%) but increased during the experiment and reached about 75% by 33 dah.

The number of snapper larvae with food in their gut ranged from 90% to 97% and did not vary significantly between physical parameter treatments for any time of sampling ($P > 0.05$).

4.5. Discussion

The "new" environmental culture regime of salinity, temperature and photoperiod (Treatment 2: 25‰, 24°C and 12L:12D to swimbladder inflation then 18L:06D) resulted in increased larval growth and development (as defined by the developmental milestone of tail flexion) of juvenile snapper in commercial-scale tanks compared with that of the previous "best-practice" regime (35‰, 21°C and 14L:10D; Treatment 1). This result confirms earlier studies where individual optima for salinity, temperature and photoperiod for snapper larvae were identified in replicated experiments using small tanks (Fielder et

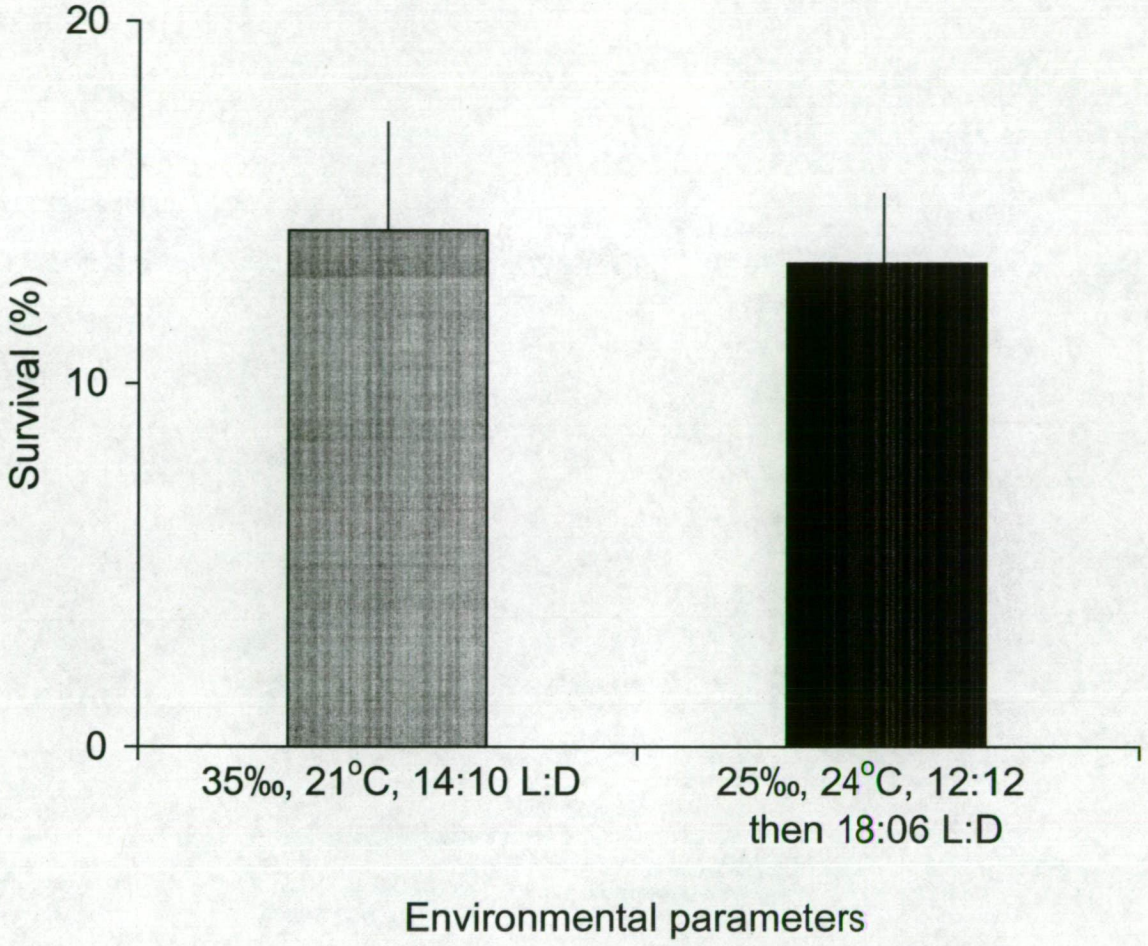

Fig. 4.4. Mean survival of of 33 dah snapper, *Pagrus auratus*, larvae grown in 2,000-l commercial tanks under different salinity, temperature and photoperiod regimes. Data are means ± S.E. for n=3 tanks. Data are not significantly different ($P > 0.05$).

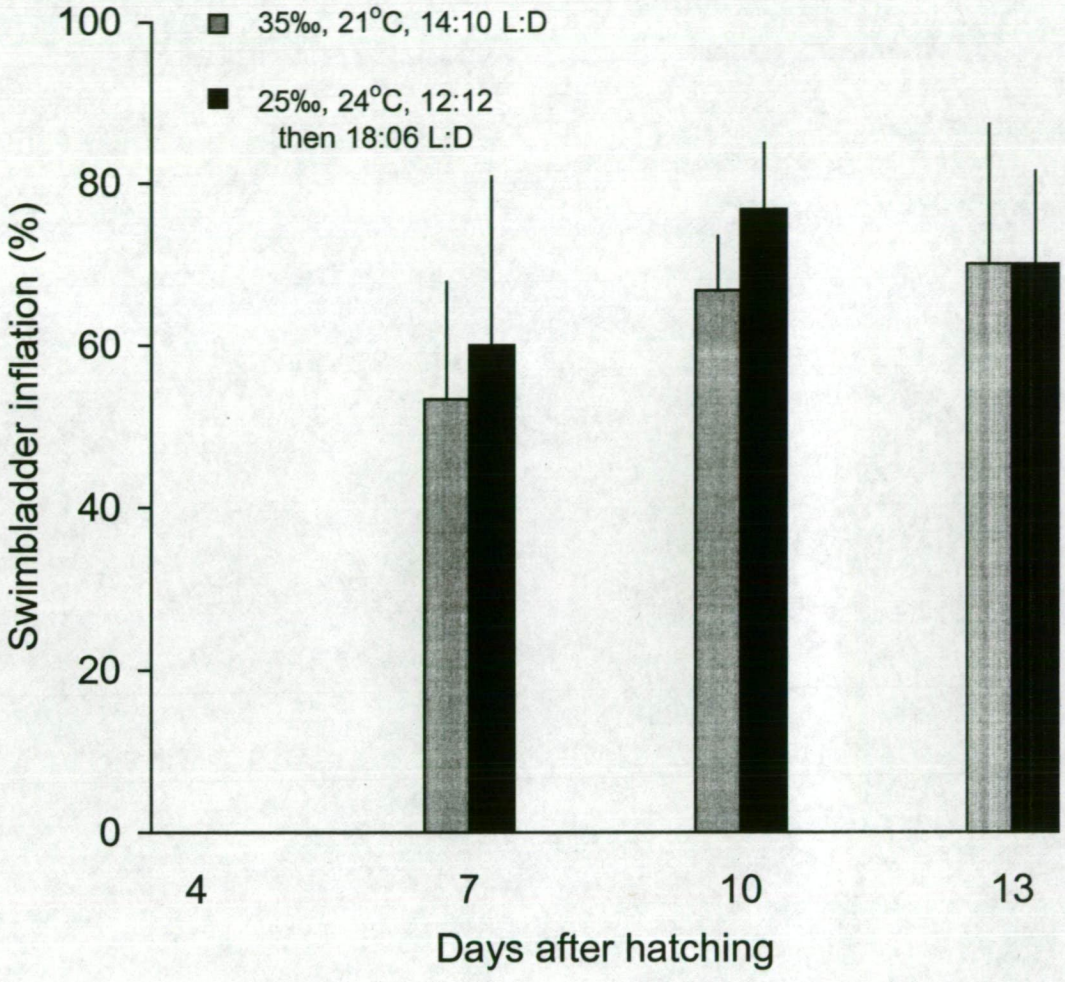

Fig. 4.5. Mean number of snapper, *Pagrus auratus*, larvae with inflated swimbladders grown in 2,000-l commercial tanks from 4 to 13 dah under different salinity, temperature and photoperiod regimes. Data are means ± S.E. for n=3 tanks. At each sampling time, bars are not significantly different ($P > 0.05$).

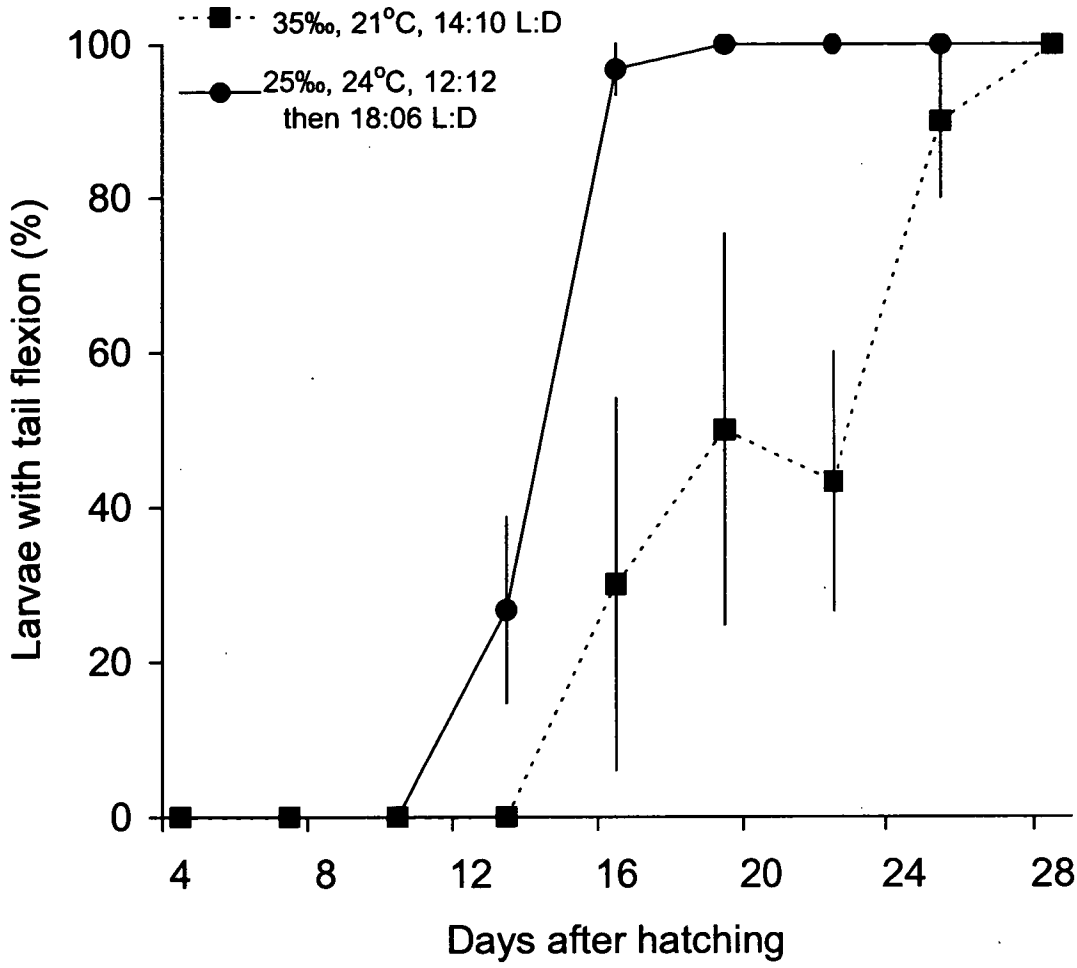

Fig. 4.6. Mean number of snapper, *Pagrus auratus*, larvae with tail flexion grown in 2,000-l commercial tanks from 4 to 33 dah under different salinity, temperature and photoperiod regimes. Data are means ± S.E. for n=3 tanks.

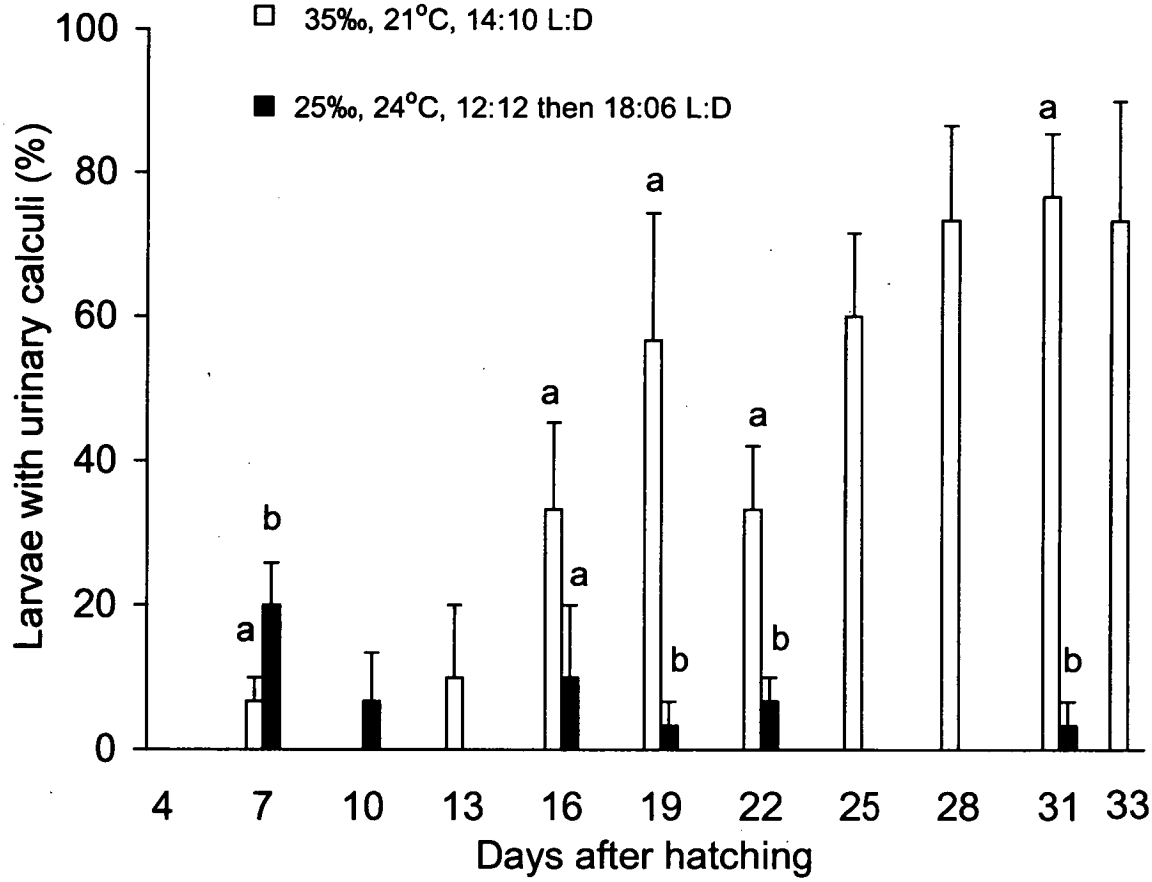

Fig. 4.7. Mean number of snapper, *Pagrus auratus*, larvae with urinary calculi grown in 2,000-l commercial tanks from 4 to 33 dah under different salinity, temperature and photoperiod regimes. Data are means ± S.E. for n=3 tanks. At each sampling time, bars with a different superscript are significantly different ($P < 0.05$).

al., 2002; Chapter 2 and 3), and supports the fact that it is essential to determine the optimal environmental requirements for larvae in order to maximise hatchery production (Tandler et al., 1995; Hart et al., 1996; Howell et al., 1998).

At 33 dah, snapper grown in the "new" regime were suitable for transfer from the intensive hatchery to a larger-scale nursery facility as they were fully-weaned from live feeds onto artificial pellet, and were 1.4-fold longer (15.6 mm TL) and 3.4-fold heavier (42.4 mg wet weight; 7.3 mg dry weight) than larvae in the "best-practice" regime, which still required live feeds. Snapper had metamorphosed and were fully squamated by 33 dah in the "new" regime (this occurs when larvae are 11-14 mm TL; Fukuhara, 1985; Battaglene and Talbot, 1992) and as such are more tolerant of a wider range of environmental factors (Lagler et al., 1962; cited in Foscarini, 1988), which may be experienced in nursery facilities.

The TL of snapper larvae at 33 dah in the "new" regime in this study was approximately 1.2-fold greater than that of larvae reared in a previous study in 2000-l and 5000-l rearing tanks (Battaglene and Talbot, 1992) in which live feeds were fed at concentrations similar to those in our study and environmental parameters were equivalent to those of the "best-practice" treatment applied in the present study. Growth of snapper larvae was lower in the study of these authors', despite a lower final stocking density of fish (0.6-1.5 fish l^{-1}) and therefore availability of more live feed for each larva than in our study (3-6 fish l^{-1}). Our results suggest that the new combination of salinity, temperature and photoperiod had a more potent effect on larval performance than density of fish and food.

Detection of treatment differences was more sensitive when wet and dry weights of snapper larvae were analysed compared with that of TL. This most likely reflects the

increase in biomass as larvae develop due to formation of ossified body parts, internal organs, musculature and fins (Matsuoka, 1982; Foscarini, 1988; Pankhurst et al., 1991).

Survival of larvae was not significantly affected by environmental rearing regime, but power of the experiment to detect treatment differences was very low (0.1) due to high variability within treatments. It would therefore be imprudent to accept the null hypothesis that environmental parameters do not affect survival of snapper. Power analysis (Searcy-Bernal, 1994) showed that more than 100 replicate tanks are needed to detect the observed difference between maximum and minimum survival means of 0.9% with power of at least 0.8. We used large, 2000-l replicate rearing tanks, which were held in a controlled environment room and managed in ostensibly the same manner, in an effort to reduce within-treatment variability. However, clearly survival of snapper larvae is a highly variable parameter and may be influenced significantly by subtle differences in physical parameters such as water flow, turbulence and aeration, among others. Nevertheless, survival to 33 dah in replicate tanks for each treatment was reasonable and ranged from 10-20%. Similarly, survival of the closely related sparid, gilthead sea bream, *Sparus aurata*, larvae to 32 dah in commercial tanks of 400-l to 1700-l tanks is consistently 20-40% (Tandler, 1993).

The number of larvae with inflated swimbladders was large and was not different between the environmental rearing regimes. This confirms that salinity of 25‰ and 35‰, temperature within the range of 21-24°C and photoperiod within the range of 12L:12D to 14L:10D, is optimal for inflation of snapper larvae swimbladders (Fielder et al., 2002; Chapter 2 and 3). Inflation of swimbladders is essential for cultured physoclistous fish to develop normally (Battaglene, 1995) and to avoid problems such as reduced growth rates (Battaglene and Talbot, 1990, 1992), spinal-deformities (Chatain, 1994; Kitajima et al., 1994; Trotter et al., 2001) and poor survival (Chatain, 1986, 1987; Chatain and Dewavrin,

1989). The ability for larvae to inflate their swimbladders can be influenced by salinity (Battaglene and Talbot, 1990; Battaglene, 1995; Tandler et al., 1995; Howell et al., 1998), temperature (Hadley et al., 1987; Gibson and Johnston, 1995; Trotter, in press) and photoperiod (Ronzani-Cerqueira and Chatain, 1991; Battaglene, 1995, Fielder et al, 2002, Chapter 2).

The presence of calculi in the urinary bladder and kidneys of gilthead sea bream (Modica et al., 1993) and red sea bream (Ueda et al., 1970) was associated with swimbladder dysfunction and may indicate that larvae were reared in sub-optimal environmental conditions. In our study, although more larvae had calculi in the urinary bladder to 10 dah in the "new" regime than in the previous "best-practice" regime, by 22 dah when larvae were 8.6 mm TL, virtually no larvae in the "new" regime had urinary calculi. In contrast, many larvae reared in the previous "best-practice" regime developed urinary calculi by 19 dah, and at 33 dah, when larvae were 11.1 mm TL, nearly all larvae had urinary calculi. The incidence of urinary calculi in snapper increases as salinity and temperature are increased (Chapter 3). In the present study, salinity had a greater effect on incidence of urinary calculi than temperature. In the "new" regime the incidence of urinary calculi decreased when salinity was decreased from 35‰ to 25‰ even though temperature was highest at 24°C. Urinary calculi have been associated with mortality of 55 dah gilthead sea bream (Modica et al., 1993), but in our study, the presence of urinary calculi was not associated with larval mortality. Similar results were reported for snapper (Battaglene, 1995) and sharpsnout seabream, *Diplodus puntazzo*, (Favaloro and Mazzola, 2000).

Many studies have investigated the effects of salinity and temperature (alone or in combination) (e.g. Kinne, 1963, Blaxter, 1969; Mihelakakis and Kitajima, 1994; Tandler et al., 1995; Hart et al., 1996; Peterson et al., 1996; Rombough, 1996), and photoperiod (Peguin, 1984, cited in Tandler, 1993; Tandler and Helps, 1985; Ronzani Cerqueira and

Chatain, 1991; Boeuf and Le Bail, 1999; Fielder et al., 2002, Chapter 2) on performance of eggs, larvae and juvenile marine fish. However few studies have determined the optimal regime for larval rearing (Tandler et al., 1995) and then evaluated this regime in commercial-scale tanks. An optimal rearing protocol for gilthead sea bream larvae to 32 dah has been developed after rigorous investigations of the main effects of salinity, temperature and photoperiod and is now routinely used in commercial cylindro-conical tanks of 400-l to 1,700-l (Tandler, 1993). The rearing regime for that sparid, gilthead sea bream is similar to that in our study; salinity reduced from initial ambient salinity of 40‰ to 25‰; temperature initially 19±0.5°C but then gradually increased to 24.5±0.5°C by 23 dah; and a photoperiod of 15L:09D.

It is important to validate results of experiments, which may have been done in tanks as small as 1-l to 3-l (Perschbacher et al., 1990; Parado-Estepa, 1991; Hart et al., 1996; Bolla and Ottesen, 1998; Specker et al., 1999), in "real" commercial-scale trials to obtain information on potential production and hatchery costs as well as necessary infrastructure and the logistics of scaling-up from experimental to commercial production. Extrapolation of the snapper larval growth curves in our study for the previous "best-practice" larval rearing regime predicts that a further 15-18 days, or approximately 1.5-times longer, will be required until these larvae attain the same size and development of larvae reared in the "new" regime. Moreover, it will be necessary to feed artemia to larvae for a further 7-8 days in the previous "best-practice" regime. These factors have major implications for the potential number of larvae produced and the cost of production of juvenile snapper each year.

It is now possible to spawn snapper year-round using truncated phototherm cycles (Fielder et al., 1999). Specific costs of operating intensive marine fish hatcheries are likely to vary between facilities, however, the major operating costs, in order of magnitude, are

labour, live feeds (artemia), artificial weaning pellets and electricity. For gilthead sea bream and sea bass, *Dicentrarchus labrax* hatcheries, these costs are estimated at approximately 35%, 17%, 16% and 11%, respectively, or 79% of the total operating costs (Candreva et al., 1996). A 1.5-times increase in larval snapper growth rate means that fish can be transferred earlier from the hatchery to the nursery and the larval rearing tanks then used for another hatchery cycle. Approximately eleven hatchery cycles are possible with the "new" regime compared with seven hatchery cycles for the previous "best-practice" regime. In addition, labour costs associated with artemia production will be reduced because bigger, faster-growing larvae can be weaned earlier from live feeds onto a pellet diet (Candreva et al., 1996).

In conclusion, snapper larvae reared in the "new" regime of 25‰, 24°C and 12L:12D to swimbladder inflation, then 18L:06D, grew more quickly than larvae in the previous "best-practice" regime and were fully weaned from live feeds to pellet diet by 33 dah and were consequently suitable for transfer from the intensive hatchery to an outdoor nursery. Potential production of snapper grown in the "new" regime can be increased by approximately 1.5-fold due to increased turnover of larvae from the hatchery and as a result costs of juvenile snapper production will be reduced.

4.6. Acknowledgements

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CHAPTER 5

Survival and growth of Australian snapper, *Pagrus auratus*, in saline groundwater from inland New South Wales, Australia

5. Survival and growth of Australian snapper, *Pagrus auratus*, in saline groundwater from inland New South Wales, Australia

5.1. Summary

Australia has extensive resources of inland saline groundwater, which may be suitable for culture of marine fish. This study assessed the suitability of saline groundwater, which was pumped from a shallow aquifer into an evaporation pond near Wakool in western New South Wales, for growth and survival of juvenile snapper *Pagrus auratus*. Five experiments were conducted. The first showed that snapper (31 g) did not commence feeding, lost equilibrium of buoyancy and became moribund within 3 days after transfer from coastal seawater (diluted to 19‰ with rainwater) to saline groundwater (19‰). Potassium concentration of diluted coastal seawater and groundwater (both 19.6‰) was 203 and 9.2 mg l^{-1}, respectively, while the concentration of most other major ions was similar in water from both sources. In the second experiment groundwater of 21‰ salinity was fortified with potassium (as KCl) to provide 25, 50 or 100% of the concentration of potassium found in coastal seawater of 21‰ salinity. Survival and feeding and swimming behaviour of snapper (1.5 g) held in tanks for 8 days were the same in 50 and 100% potassium fortified treatments as in coastal seawater controls. However, snapper held in groundwater fortified with only 25% potassium, or raw saline groundwater became moribund after 4 and 2 days, respectively. During the third 42 d experiment, growth, survival and food conversion of juvenile snapper (4.0 g) were the same in diluted coastal seawater (20‰) and groundwater (20‰) provided the level of potassium in the groundwater was increased to within 60-100% of the concentration in coastal seawater. During the fourth experiment, juvenile snapper were acclimatised to raw saline groundwater by transferring fish from fortified groundwater with initial potassium levels of

100% of that in coastal seawater, to groundwater with 10% lower potassium levels every 3.5 days or 20% lower levels every 7 days. A further treatment where snapper were transferred from groundwater fortified initially with potassium levels of 60% of coastal seawater, to groundwater with 20% lower potassium levels every 3.5 days was included. When potassium was reduced to 20% of the concentration in coastal seawater, in all treatments, fish became moribund. Results from the fifth experiment, where groundwater was fortified with either KCl or NaCl at equivalent chloride levels, confirmed that potassium and not chloride ions were responsible for improvement in groundwater. Our results demonstrate that saline groundwater from Wakool, fortified with KCl is a suitable medium for growing snapper juveniles in tanks.

5.2. Introduction

Snapper, *Pagrus auratus*, occurs in temperate waters in all Australian states, Lord Howe Island and Norfolk Island (Battaglene and Bell, 1991) and is the target of recreational and commercial fisheries (Bell et al., 1991; Francis, 1994). Juveniles usually live in estuaries, while adults inhabit coastal and offshore waters. Catches, particularly from the Australian east-coast are declining (ABARE, 1998) and as a consequence programs to develop aquaculture protocols for snapper are current in temperate Australia (Battaglene and Fielder, 1997).

Australian snapper is the same species as the Japanese red sea bream (*P. auratus* = *P. major*; Paulin, 1990), which has been cultured successfully in Japan for 30 years, using intensive larval rearing followed by growout in sea-cages (Foscarini, 1988; Battaglene and Bell, 1991). It is seen as an excellent candidate species for aquaculture and possibly stock enhancement in Australia.

Commercial snapper hatcheries and floating sea-cage farms are now operating in New South Wales (NSW), South Australia and Queensland using techniques described by Battaglene and Talbot (1992), Battaglene (1995) and Quartararo (1996) or adapted from those used in Japan to rear red sea bream. However, expansion of a sea-cage-based snapper farming industry in Australia may be limited by the lack of sites with suitable water quality, water depth and proximity to land-based infrastructure (Ogburn, 1996). Approval of sea-cage farms by relevant government bodies is also affected by conflict with other waterway users and perceived concerns about environmental impact.

Alternative, additional sites may be available for farming marine species if the large reserves of saline groundwater occurring in inland areas of Australia can be utilised. Shallow saline water tables are migrating towards the soil surface in many semi-arid areas especially where crops are irrigated (Blackwell, 1999). Crop production is affected

adversely and natural vegetation is destroyed. In some inland areas upward migration of the water table is addressed by pumping saline groundwater into a series of large evaporation ponds, which range in size from 2-30 ha (Ingram et al., 1996; Allan and Fielder, 1999). The salt concentration of this water increases progressively as it flows from one pond to the next and eventually crystalline salt is deposited.

The largest saline groundwater evaporation scheme in Australia is the Wakool-Tullakool Sub-Surface Drainage Scheme at Wakool, NSW where approximately 13,000 Ml yr^{-1} of saline groundwater is pumped into 1,600 ha of evaporation ponds (Ruello, 1996). The major ions found in seawater, in order of magnitude, are chloride (Cl^-), sodium (Na^+), sulphate (SO_4^{2-}), magnesium (Mg^{2+}), calcium (Ca^{2+}) and potassium (K^+) (Spotte, 1979). In saline groundwater from Wakool, the concentration of Cl^-, Na^+, SO_4^{2-} and Mg^{2+}, is similar to seawater of the same salinity; however, the concentration of Ca^{2+} and K^+ in Wakool saline groundwater is much higher and lower, respectively, than seawater of the same salinity.

Saline groundwater has been used successfully in the United States and the Middle East to culture a range of algae, crustaceans and finfish such as tilapia, red drum, sea bream, eels and channel catfish (Forsberg et al., 1996; Ingram et al., 1996; Samocha et al., 1998). In Australia, saline groundwater from shallow and deep aquifers has been suitable for growth and survival of euryhaline finfish such as silver perch, *Bidyanus bidyanus*, Australian bass, *Macquaria novemaculeata*, barramundi, *Lates calcarifer* and Atlantic salmon, *Salmo salar* (Allan and Fielder, 1999; Gooley et al., 1999). To date, attempts have not been made to culture relatively stenohaline marine finfish like snapper in Australian saline groundwater.

The aim of this study was to assess the suitability of saline groundwater collected from an evaporation pond at Wakool, for growth and survival of juvenile snapper.

5.3. Materials and Methods

Five laboratory bioassays were conducted at the NSW Fisheries Port Stephens Fisheries Centre (PSFC) from July 1997 to April 1998.

5.3.1. Source of juvenile snapper

Fertilised snapper eggs were obtained from first generation hatchery-reared (G1) snapper held in tanks at PSFC. Snapper either spawned spontaneously or were induced to spawn using hormones and techniques described by Lee et al. (1986).

The first group of juvenile snapper was produced after larvae were cultured for 57 days in a 2,000-l tank using techniques similar to those described by Battaglene and Talbot (1992). After this time, approximately 1,000 juvenile snapper were harvested from the tank and 200 were placed into each of five, 500-l floating cages placed within a 100,000-l, outdoor, in-ground concrete tank. While in the cages, juvenile snapper were fed a 45% protein, 1-4 mm crumble diet (Kinta Pty Ltd, Australia). Ambient estuarine water (range, 23-33‰, 14-25°C) was pumped into the tank for 4 h each day to replace approximately 20% of the total tank volume.

The second group of juvenile snapper was produced after larvae were cultured for 32 days in three 10,000-l outdoor, concrete tanks using "greenwater" culture techniques. After this time, juvenile snapper were harvested and ongrown in 10,000-l fibreglass tanks until used in experiments. Estuarine seawater (30-35‰) was supplied constantly with a flow-through rate of approximately 200 l h^{-1}. While in the tanks, juvenile snapper were fed 400 and 800 μm, 52% protein "ML powered" weaning diet.

5.3.2. Source of saline groundwater and coastal seawater

Saline groundwater was collected on two occasions from an individual 30 ha evaporation pond which was part of the Murray Irrigation Limited, Wakool-Tullakool Sub-Surface Drainage Scheme (35°28'S, 144°26'E) and transported in tanks by road to the PSFC. The saline groundwater collected first (collection 1) had a salinity of 19‰ and was stored until needed for experiments in 10 x 30-l drums. The salinity increased to 21‰ following evaporation during this study. The saline groundwater collected second (collection 2) had a salinity of 30‰ and was stored in a 10,000-l storage tank which was covered by a lid to exclude light and reduce evaporation.

Coastal seawater was collected from an ocean beach at Port Stephens (32°45'S, 152°04'E), transported by road to the PSFC and stored in a 10,000-l tank.

Generally, the chemistry of the saline groundwater (SG) from Wakool and coastal seawater (CS) of the same salinity was similar (Table 5.1). Both SG and CS were dominated by chloride ions, which constituted approximately 56% of the total salinity. Of the other major elements, in order of magnitude, the concentrations of sodium and sulphate in SG were 80% of that in CS, whereas the concentrations of magnesium and calcium were 1.5 and 2.5 times greater, respectively in SG than in CS. There was a major difference in the concentration of potassium, which was present in SG at only 4.5% of the concentration in the same salinity CS. There were mostly small differences in concentration of minor elements, such as heavy metals, in SG and CS (Table 5.1).

5.3.3. Experiment 1: effect of raw saline groundwater

This experiment was designed to determine the effect of raw saline groundwater on short-term survival and behaviour of juvenile snapper. Two water treatments were tested: raw saline groundwater (SG) and coastal seawater (CS).

Table 5.1.
Water chemistry of coastal seawater and saline groundwater from the Wakool-Tullakool Sub Surface Drainage Scheme, New South Wales, Australia.

Element or Chemical	Coastal seawater		Coastal seawater diluted[1]		Raw groundwater	
Salinity (‰)	35.3		19.6		19.6	
pH	8.1		--		7.9	
Alkalinity, (mg l^{-1}) as $CaCO_3$	114		63		195	
Major ions	(mg l^{-1})	ion/Cl^- ratio	(mg l^{-1})	ion/Cl^- ratio	(mg l^{-1})	ion/Cl^- ratio
Chloride	20000	1.000	11105	1.000	11000	1.000
Sodium	9470	0.474	5258	0.474	4210	0.383
Sulphate	2500	0.125	1388	0.125	1100	0.100
Magnesium	1000	0.050	555	0.050	820	0.075
Potassium	365	0.018	203	0.018	9.2	0.001
Calcium	364	0.018	202	0.018	504	0.046
Minor elements						
Fluoride	0.76		0.42		0.44	
Total PO_4, (mg l^{-1}) as P	< 0.10		--		< 0.10	
Aluminium	< 0.001		--		0.007	
Boron	4.4		2.4		0.38	
Bromide	72		40		34	
Copper	0.012		0.007		0.007	
Iodide	< 0.05		--		0.16	
Iron	< 0.05		--		< 0.05	
Lead	< 0.001		--		< 0.001	
Lithium	0.17		0.09		0.07	
Manganese	< 0.001		--		0.001	
Mercury	< 0.001		--		< 0.001	
Molybdenum	0.012		0.007		0.002	
Strontium	7.6		4.2		9.1	
Zinc	0.004		0.002		< 0.001	

[1]values calculated from coastal seawater at 35.3‰.

The experiment was conducted in 6 x 60-l glass aquaria (three replicates per treatment). Each aquarium had a simple airlift-driven biofilter, which occupied approximately 20% of the volume. Tanks were covered with black plastic to reduce light intensity, but a window covered by a black plastic flap, allowed observation of experimental fish.

Three randomly positioned tanks were filled with each experimental treatment solution (Table 5.2). Each tank was then stocked with four randomly selected juvenile snapper (mean wet weight 31.3 g, $n = 10$; group 1), which had been acclimatised from 30 to 19‰, 24 h earlier. No water was exchanged during the experiment, which was terminated after 4 days.

5.3.4. Experiment 2: short-term effect of fortified saline groundwater

This experiment was designed to determine the effect of fortifying raw saline groundwater with K^+ (as KCl) on short-term survival and behaviour of juvenile snapper. For this and subsequent experiments, stock solutions for experimental treatments which included K^+ fortification, were prepared by dissolving analytical grade KCl to give the required K^+ concentration (Table 5.2). Coastal seawater was diluted using rainwater. The following water treatments (all 21‰) were tested:

(a) raw saline groundwater (SG raw)

(b) 100% K^+ fortified saline groundwater (SG100)

(c) 50% K^+ fortified saline groundwater (SG50)

(d) 25% K^+ fortified saline groundwater (SG25)

(e) coastal seawater control (CS).

Table 5.2.

Experimental water treatments, target ion concentrations and quantities of added salts (KCl; NaCl). Coastal seawater (CS) and saline groundwater (SG) were collected from Port Stephens and Wakool, New South Wales, Australia, respectively.

	Treatment	K^+ concentration ($mg\ l^{-1}$)	Amount of KCl added ($mg\ l^{-1}$)
Experiment 1	CS[a]	197	0
(19.0[b] ‰)	SG raw	9	0
Experiment 2	CS[a]	217	0
(21.0[b] ‰)	SG100	217	395
	SG50	109	188
	SG25	54	84
	SG raw	10	0
Experiment 3	CS[a]	207	0
(20.0[c] ‰)	SG100	207	378
	SG80	166	300
	SG60	124	220
	SG40	83	141
	SG100-30[d]	310	565
Experiment 4	SG100	223	407
(21.5[c] ‰)	SG90	200	363
	SG80	178	321
	SG70	156	279
	SG60	134	237
	SG50	112	195
	SG40	90	153
	SG30	67	109
	SG20	45	67
	SG10	22	23
	SG raw	10	0

		K^+ concentration ($mg\ l^{-1}$)	added Cl^- concentration ($mg\ l^{-1}$)	Amount of KCl added ($mg\ l^{-1}$)	Amount of NaCl added ($mg\ l^{-1}$)
Experiment 5	SGK100	207	188	395	0
(21.0[c] ‰)	SGCL100	10	188	0	310

[a]Coastal seawater diluted with rainwater from 35.3 ‰.

[b]Undiluted saline groundwater; collection 1.

[c]Saline groundwater diluted with rainwater from 30 ‰; collection 2.

[d]Undiluted saline groundwater; collection 2.

The experiment was conducted in 15 x 2-l opaque, plastic buckets with lids (three replicates per treatment). A total of 1.5 l of each experimental water treatment was placed into three randomly selected buckets. Approximately 85% of the bucket water volume was poured to waste each day and replaced with new treatment water. Air was supplied to each container through a 1-ml glass pipette at 50 ml min^{-1}. Fluorescent light was provided at the surface of each container at 3.0 µmol s^{-1} m^{-2} on a 14:10 h light:dark photoperiod.

Each bucket was stocked with 4 randomly selected 61 dah juvenile snapper (mean wet weight 1.5 g, $n = 4$; group 2), which had been acclimatised from 30 to 21‰, 24 h earlier. The experiment was terminated after 8 days.

5.3.5. Experiment 3: long-term effect of fortified saline groundwater

This experiment was designed to determine the effect of fortifying raw saline groundwater with K^+ on long-term survival and growth of juvenile snapper. The water treatments (all 20‰, unless otherwise stated; Table 5.2) tested were:

(a) 100% K^+ fortified saline groundwater (SG100)

(b) 80% K^+ fortified saline groundwater (SG80)

(c) 60% K^+ fortified saline groundwater (SG80)

(d) 40% K^+ fortified saline groundwater (SG40)

(e) 100% K^+ fortified saline groundwater, 30‰ (SG100-30)

(f) coastal seawater control (CS).

The experiment was conducted in 24 x 100-l conical bottomed tanks with black sides and white bottoms (four replicates per treatment). Each tank was part of an independent, recirculating system operated with an internal 500-µm mesh-covered standpipe, an external airlift pump and biofilter (described by Fielder and Bardsley, 1999) and an internal, fully-immersed, 2.75-l airlift-driven biofilter, which was filled with bioballs (Academy Plastics,

Australia). Incandescent light was provided at the surface of each tank at 23 ± 1.3 μmols^{-1}m^{-2} (mean ± S.E.; n = 30 tanks) on a 14:10 h light:dark photoperiod. Approximately 5% of the experimental tank water was exchanged each day and salinity was maintained by adding rain water when needed.

Each experimental water treatment was placed into four randomly selected tanks. Juvenile snapper (97 dah; group 2) were anaesthetised with 20 mg l^{-1} of benzocaine in the stock tank, and a random sample of fish was weighed to provide an estimate of fish weight (3.9 ± 0.8 g, ± S.D., n = 20). Fish were then weighed individually and 6 fish of similar size were stocked into each experimental tank. Fish were fed to satiation daily by hand at 0900 and 1500 h with 52% protein "ML powered" weaning pellets. The experiment was terminated after 42 d.

5.3.6. Experiment 4: acclimation of juveniles to raw saline groundwater

This experiment was designed to determine if juvenile snapper could be acclimatised from KCl fortified saline groundwater to raw saline groundwater by rapid or slow dilution of the K^+ concentration. The acclimation treatments (all 21.5‰; Table 5.2) tested were:

(a) 100% K^+ fortified saline groundwater; no reduction in K^+ (SG100-C)

(b) 100% K^+ fortified saline groundwater; K^+ reduced by 10% every 3.5 days (SG100-slow)

(c) 100% K^+ fortified saline groundwater; K^+ reduced by 20% every 7 days (SG100-rapid)

(d) 60% K^+ fortified saline groundwater; K^+ reduced by 20% every 7 days (SG60-rapid).

The experimental buckets, daily water management and lighting for this experiment were the same as those described for Experiment 2. Each experimental water treatment was placed into three randomly selected buckets. Three randomly selected 109 dah juvenile

snapper (mean wet weight 3.2 ± 0.6 g, ± S.D., n = 10; group 2) were then placed into each bucket. The experiment was terminated after 33 days.

5.3.7. Experiment 5: effect of K^+ or Cl^-

This experiment was designed to determine whether the improvement in performance of juvenile snapper in raw saline groundwater fortified with KCl was due to an increase in K^+ or Cl^- concentration. The water treatments (21‰; Table 5.2) tested were:

(a) 100% K^+ fortified saline groundwater (SGK100)

(b) saline groundwater fortified with analytical grade NaCl to provide the same Cl^- concentration as occurred in treatment (a) (SGCL100).

The experiment was conducted in 6 x 4-l glass beakers (three replicates per treatment), each filled with 3.4 l. The daily water management and lighting were the same as those described for Experiment 2. Each experimental water treatment was placed into three randomly selected beakers. Each tank was then stocked with 3 randomly selected 168 dah juvenile snapper (mean wet weight 21.9 ± 4.5 g, ± S.D., n = 6 tanks; group 2). The experiment was terminated after 4 days.

5.3.8. Measurement of survival, fish behaviour, growth and feeding

For experiments 1, 2, 4 and 5 snapper were fed to satiation by hand with 54% protein "ML powered" pellets once each day at approximately 0900 h when the number of surviving fish and the number of fish feeding were counted, and swimming behaviour was assessed. Moribund and dead fish were removed from the tanks as soon as they were noticed. In experiments 2, 4, and 5 the feeding and swimming behaviour of individual fish was scored on a scale of 5 (normal) to 1 (abnormal) (Table 5.3).

In experiment 3, all moribund and dead fish were removed from the tanks as soon as they were noticed. Stocking density was maintained in each tank by replacing dead fish with fin-clipped fish of similar weight. A group of replacement fish for this purpose were pelvic fin-clipped for identification 7 d before the start of the experiment, treated for 48 h with 50-100 mg l^{-1} of oxytetracycline hydrochloride to prevent infection of wounds and then held in a 400-l tank with flow-through estuarine seawater (30-32‰). Replacement fish were not used in estimates of weight gain. Feed consumption was recorded daily. Weight gain was recorded every 14 d. After 42 d, fish were harvested and the percentage survival, mean fish wet weight, mean adjusted biomass gain = [final total biomass + weight of dead fish] – [initial total biomass + weight of replacement fish], mean fish dry weight and food conversion ratio (FCR) were calculated from data recorded for each tank.

5.3.9. Daily water measurement

In all experiments salinity, temperature, pH and dissolved oxygen were measured daily using a water quality meter (Horiba U-10, Japan) (Table 5.4). In Experiment 3, Total ammonia-nitrogen (< 1.0 mg l^{-1}) was measured daily in at least one replicate tank from each treatment with a rapid test kit (E. Merck, Model 1.08024, Germany). Light intensity (photosynthetically active radiation) was measured with a light meter (LI-COR, model Li-1776, USA). Chemical analyses of treatment water was done by a National Accredited Testing Agency, Hunter Water Laboratories (A Division of Hunter Water Corporation Ltd), Newcastle, Australia.

5.3.10. Statistical analyses

Data were assessed for homogeneity of variance using Cochran's test (C; Winer, 1971). For Experiment 3, data for survival ($P = 0.0001$, $C = 0.8$) and FCR ($P = 0.003$, $C = 0.7$) were heterogeneous and could not be transformed to satisfy the assumption of homogeneity of variance. Experiments 2-5 were designed for analysis using single factor analysis of variance (ANOVA). Where significant differences were found, means were compared by the Student-Newman-Keuls test (SNK). Statistical analyses were conducted using Statgraphics Version 5.0 (STSC Inc., USA).

Table 5.3.

Feeding and swimming behaviour criteria and scoring of juvenile snapper, *Pagrus auratus* in saline groundwater. Experiments 2, 4 and 5.

Feeding Behaviour	
Score	
5	fish respond immediately to introduction of pellet to tank; vigorous rate of feed ingestion
4	fish respond immediately to introduction of pellet to tank; moderate rate of feed ingestion
3	fish respond immediately to introduction of pellet to tank; slow rate of feed ingestion
2	fish respond slowly to introduction of pellet to tank; slow rate of feed ingestion
1	fish do not respond to introduction of feed pellet to tank; no feed ingested

Swimming Behaviour	
Score	
5	fish upright (normal orientation); highly alert to external stimuli; active swimming throughout water column
4	fish upright (normal orientation); moderately alert to external stimuli; active swimming throughout water column
3	fish upright (normal orientation); moderately alert to external stimuli; lethargic swimming throughout water column
2	fish upright (normal orientation); no response to external stimuli; lethargic swimming near bottom of tank
1	fish moribund; lying over on tank bottom or floating upside down

Table 5.4.
Ranges of water quality parameters for Experiments 1-5.

Experiment	DO_2 ($mg\ l^{-1}$)	Salinity (‰)	pH	Temperature (°C)
1	5.6-6.4	18.0-19.0	8.3-8.5	20.5-21.6
2	4.0-6.0	20.8-21.5	7.4-8.7	20.3-24.2
3	6.2-7.9	19.7-20.9 (target 20) 29.7-31.1 (target 30)	7.4-8.1	22.1-24.4
4	4.0-7.5	21.5-23.3	7.7-8.1	21.9-22.7
5	5.1-6.9	21.1-21.7	7.7-8.1	21.9-22.6

5.4. Results

5.4.1. Experiment 1: effect of raw saline groundwater

Snapper held in SG were actively swimming for 1 d following transfer from CS, but did not feed vigorously. After 2 d, one fish had died and two fish had lost equilibrium of buoyancy and were floating upside down, while the remaining fish were lethargic and displayed no flight response when challenged with external stimuli. This trend continued, and after 4 d all fish in the SG were removed from treatment tanks following loss of equilibrium of buoyancy, or had died. Snapper held in CS treatments for 4 d all survived, and were actively swimming and feeding during this time.

Effort was made in this and all subsequent experiments to remove moribund snapper from the treatment solutions and soon as they were noticed, and return them to coastal seawater; however, some snapper died prior to removal from treatment solutions and no fish survived after transfer to seawater.

5.4.2. Experiment 2: short-term effect of fortified saline groundwater

After 8 d, all fish that were held in CS, SG50 and SG100 treatments had survived and there was no significant difference ($P > 0.05$) in swimming and feeding behaviour (Table 5.5). No fish survived for more than 2 and 4 days when held in the SG raw and SG25 treatments, respectively.

5.4.3. Experiment 3: long-term effect of fortified saline groundwater

Survival of snapper was high and not significantly different ($P > 0.05$) for all water treatments (Table 5.6). A total of three fish died during the experiment as a result of

jumping from the tanks; one fish in one tank of the SG60 treatment, and two fish in one tank of the SG40 treatment.

Snapper grew in all water treatments; however, the final wet weight of snapper grown in the SG40 treatment, was significantly lower ($P < 0.05$) than that of snapper grown in CS, SG60, SG80, SG100 or SG100-30, which did not differ (Table 5.6). Multiple comparisons of means for final dry weight failed to clearly separate treatment differences; however, the final dry weight of snapper grown in SG40 was significantly ($P < 0.05$) lower than that of snapper grown in SG60, SG80, SG100 or SG100-30 which were similar (Table 5.6).

The amount of feed consumed and FCR were affected significantly ($P < 0.05$) by the concentration of K^+ in the saline groundwater; however, multiple comparisons of the means failed to clearly separate the main treatment effects. The fish grown in the SG40 consumed up to 28.8% less feed than snapper grown in the SG100 treatment. Feed consumption was similar for the other treatments (Table 5.6). The FCR of snapper was low and similar for fish grown in the CS, SG60, SG80, SG100 and SG100-30 treatments, but was higher for snapper grown in the SG40 treatment (Table 5.6).

5.4.4. Experiment 4: acclimation of juveniles to raw saline groundwater

All snapper survived and the feeding and swimming behaviour of the fish were similar and normal for all acclimation treatments provided the K^+ concentration was $\geq$ 40% of that in equivalent salinity CS (Fig. 5.1). However, when the K^+ concentration was diluted to 20% for the SG100-slow, SG100-rapid and SG60-rapid treatments, some fish lost equilibrium and stopped feeding within 1-2 d and all fish were moribund after 4-6 d.

Table 5.5.

Final survival, swimming and feeding behaviour[a] of juvenile snapper *Pagrus auratus* held for 8 d in saline groundwater fortified with KCl to provide potassium at different concentrations as occurred in equivalent salinity coastal seawater (Experiment 2).

Treatment	Survival (%)	Swimming behaviour[b]	Feeding behaviour[b]
Coastal Seawater	100 ± 0	5.0 ± 0	4.8 ± 0.3
SG 100% K^+	100 ± 0	4.8 ± 0.3	4.7 ± 0.3
SG 50% K^+	100 ± 0	4.8 ± 0.3	4.1 ± 0.5
SG 25% K^+	0 ± 0	0 ± 0	0 ± 0
SG raw	0 ± 0	0 ± 0	0 ± 0

Data are means ± standard errors (n = 3 tanks).

[a] feeding and swimming behaviour of individual fish was scored on a scale of 5 (normal) to 1 (abnormal).

[b] a score of 0 indicates that there were no surviving fish

Table 5.6.
Growth performance, survival and food conversion of juvenile snapper *Pagrus auratus* grown for 42 d in saline groundwater fortified with KCl to provide potassium at different concentrations as occurred in equivalent salinity coastal seawater (Experiment 3)[a].

Treatment		Salinity (‰)	Initial wet weight (g)	Final wet weight (g)	Final dry weight (g)	Adjusted wet weight gain[b] (g)	Survival (%)	Feed input[c] (g)	FCR[d]
Seawater	(CS)	20	4.1 ± 0.1^{x}	12.5 ± 0.6^{x}	3.8 ± 0.2^{y}	51.0 ± 3.4^{x}	100.0 ± 0.0^{x}	73.0 ± 1.6^{yz}	1.5 ± 0.1^{xy}
100% K^+	(SG100)	20	4.1 ± 0.1^{x}	14.8 ± 0.6^{x}	4.6 ± 0.2^{z}	64.5 ± 4.2^{x}	100.0 ± 0.0^{x}	78.7 ± 2.6^{z}	1.2 ± 0.04^{x}
80% K^+	(SG80)	20	4.0 ± 0.2^{x}	13.9 ± 0.5^{x}	4.2 ± 0.2^{yz}	59.5 ± 1.5^{x}	100.0 ± 0.0^{x}	76.4 ± 2.6^{yz}	1.3 ± 0.03^{xy}
60% K^+	(SG60)	20	4.0 ± 0.1^{x}	14.0 ± 0.6^{x}	4.3 ± 0.2^{yz}	58.9 ± 2.4^{x}	95.8 ± 4.1^{x}	77.9 ± 2.9^{z}	1.3 ± 0.03^{xy}
40% K^+	(SG40)	20	3.9 ± 0.2^{x}	10.1 ± 0.8^{y}	2.8 ± 0.2^{x}	35.1 ± 4.8^{y}	91.7 ± 8.3^{x}	56.1 ± 4.2^{x}	1.7 ± 0.2^{y}
100% K^+	(SG100-30)	30	3.8 ± 0.1^{x}	12.3 ± 0.5^{x}	3.6 ± 0.2^{y}	51.1 ± 3.4^{x}	100.0 ± 0.0^{x}	66.3 ± 2.6^{y}	1.3 ± 0.1^{xy}

[a] Data are means ± S.E. for 4 replicate tanks. Means in each column with a different superscript are significantly different ($P < 0.05$).

[b] Adjusted wet weight gain = [final total weight + weight of mortalities] - [initial total weight + weight of replacement fish].

[c] Mean total feed consumed per tank expressed as grams dry weight.

[d] Food conversion ratio = weight of feed fed/adjusted wet weight fish gain.

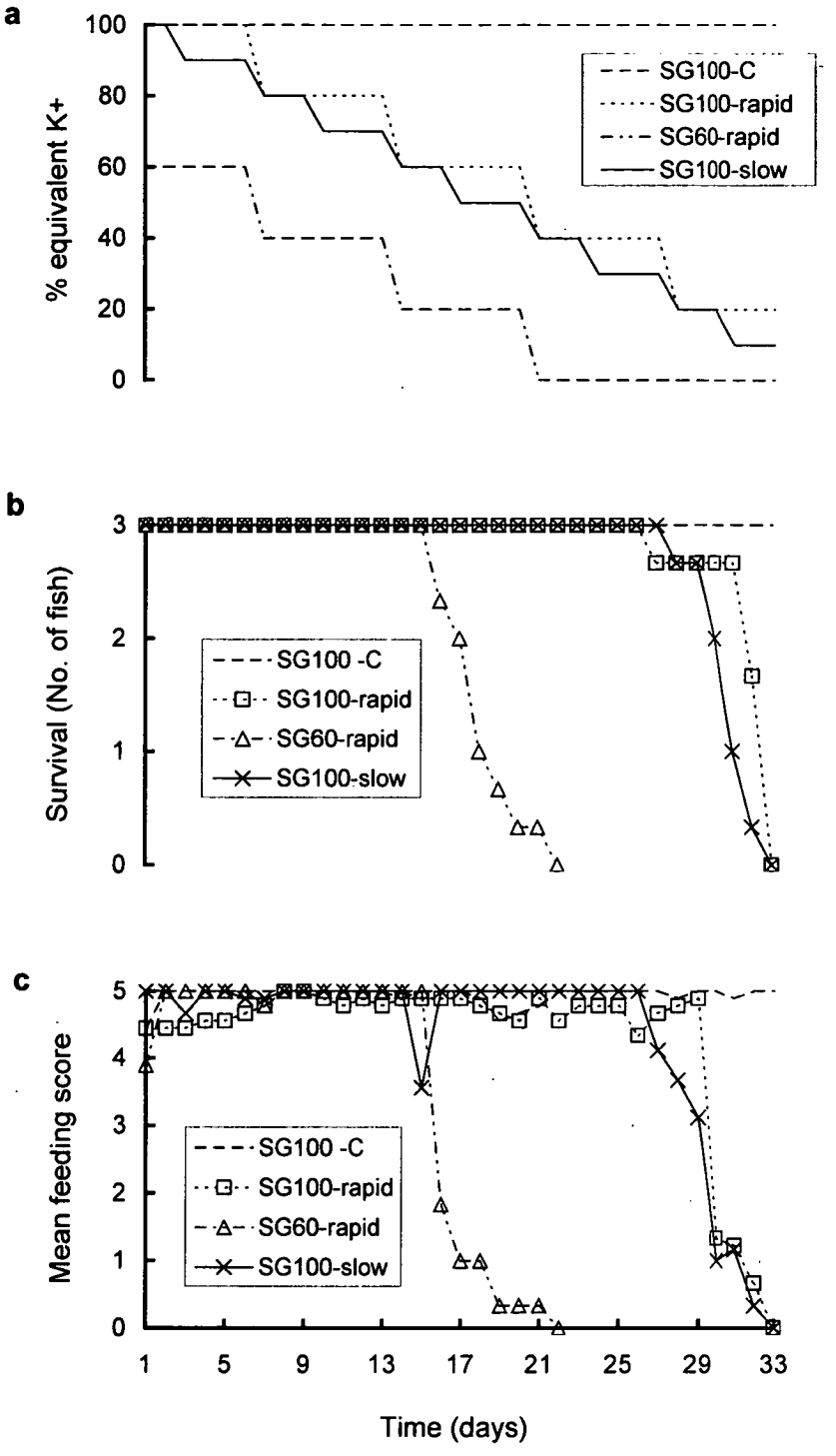

Fig. 5.1. Potassium dilution schedule [a], mean survival [b] and mean feeding score [c] of juvenile snapper *Pagrus auratus* exposed to decreasing concentration of potassium in saline groundwater from Wakool. For survival, data are means of $n = 3$ buckets (3 fish per bucket). For feeding, fish were scored from 5 (normal) to 1 (abnormal). (Experiment 4).

5.4.5. Experiment 5: effect of K^+ or Cl^-

Snapper that were held in the NaCl fortified groundwater treatment began to lose equilibrium of buoyancy and appetite after 1 d following transfer from coastal seawater. After 2 d, four fish were moribund and the remaining fish were lethargic and did not feed well. This trend continued, and after 4 d all fish in the NaCl fortified groundwater treatment were moribund. Snapper held in the KCl fortified groundwater treatment for 4 d all survived, and were actively swimming and feeding during this time.

5.5. Discussion

Raw saline groundwater from Wakool was not suitable for survival and growth of juvenile snapper. Within 1-2 d following transfer of snapper from coastal seawater (CS) to raw saline groundwater (SG), fish started to lose equilibrium of buoyancy, floated upside down and did not feed well. The performance of snapper did not improve with time and by approximately 4 d after transfer all snapper were moribund or dead.

The concentration of the major ions, Cl^-, Na^+, SO_4^{2-}, Mg^{2+} and Ca^{2+} in SG was either similar to or greater than that of equivalent salinity CS. However, the concentration of K^+ in the SG was extremely low, being only 4.5% (9.2 mg l^{-1}; 19.6‰) of that available in equivalent salinity CS (203 mg l^{-1}). The salinity and chemistry of saline groundwater can vary widely. For example, in a similar study in Texas, USA saline groundwater was collected from 35 sites and representative samples evaluated for their suitability for aquaculture of red drum, *Sciaenops ocellatus* (Forsberg et al., 1996). The concentration of the major ions were: salinity, 2–35‰; Cl^-, 639–15,443 mg l^{-1}; Na^+, 537–9,403 mg l^{-1}; SO_4^{2-}, 164–5,934 mg l^{-1}; Mg^{2+}, 8–1,263 mg l^{-1}; K^+, 5–87 mg l^{-1}; and Ca^{2+}, 36–1,179 mg l^{-1}.

Sodium, potassium and chloride are essential minerals for animals due to their role in electrolyte and acid-base balance (Wilson and El Naggar, 1992). Potassium is the main

cation of intracellular fluids while sodium and chloride are the major extracellular ions. Osmoregulation, or the maintenance of constant extracellular and intracellular osmolality, is mostly determined by the homeostasis of these ions (Teeter, 1997). Fish can readily derive all or a portion of these minerals from the water by unidirectional diffusion across the gills and the gut or they can be obtained from food (Shearer, 1988; Lall, 1989; Wilson and El Naggar, 1992). Because fish can sequester potassium from the water, it has been difficult to conduct studies to determine dietary requirement of this ion without altering the ionic composition of the water (Wilson and El Naggar, 1992). Consequently, there is a paucity of information describing the quantitative requirement of potassium (Lall, 1989).

The estuarine Japanese red sea bream, the same species as snapper (Paulin, 1990), and the freshwater channel catfish did not require dietary potassium when grown in seawater and freshwater (4 mg l^{-1} K^{+}), respectively (Sakamoto and Yone, 1978; Wilson and El Naggar, 1992), thus indicating that these fish had obtained all necessary potassium from the water. Alternatively, Shearer (1988) showed that juvenile chinook salmon, *Oncorhynchus tshawytscha* grown in freshwater with a potassium concentration of < 1 mg l^{-1}, were unable to sequester sufficient potassium from the water and required a dietary potassium concentration of 0.8% for maximum growth. Wilson and El Naggar (1992) however contend that 0.8% is an overestimate of the potassium requirement for chinook salmon. Potassium deficient chinook salmon initially displayed reduced growth rate due to anorexia and poor food conversion and ultimately fish died.

Results of our study showed that performance of snapper improved dramatically when KCl was added to the SG. The enhanced performance of fish was due to an increase in the concentration of potassium ions rather than an increase in the concentration of chloride ions (Experiment 5). In Experiment 3, growth, feed consumption and FCR after 42 d were the same for snapper held in CS and SG (both 20‰), provided the potassium concentration

of the SG was fortified to 60–100% (~ 124-207 mg l^{-1} K^+) of the potassium concentration as occurred in equivalent salinity CS. Also, the performance of snapper grown in higher salinity SG (30‰) and fortified to provide 100% (~ 310 mg l^{-1} K^+) was the same as the CS control fish. However, snapper held in SG (20‰) fortified with 40% (~ 83 mg l^{-1} K^+) of the potassium concentration as occurred in equivalent salinity CS, weighed significantly less ($P < 0.05$) and consumed almost 30% less feed and had a higher FCR than other treatments. Survival of snapper was not affected by these concentrations of potassium in SG; however, below 40% fortification of SG with potassium, fish were moribund within 2-6 d (Experiments 2 and 4).

Fortification of culture water with salts has improved growth and/or survival of fish in several other studies; however results have varied with salt and water type. Survival of red drum was markedly improved from 0 to 93% when the calcium and chloride concentrations of saline groundwater (3–4‰) were increased from 36 to 337 mg l^{-1}, and 639 to 1,296 mg l^{-1}, respectively following addition of $CaCl_2$; whereas addition of NaCl to the saline groundwater only improved survival slightly (Forsberg et al., 1996). On the other hand, addition of NaCl to freshwater and diluted seawater (1‰) resulted in much better survival (> 2-fold increase) of red drum than when $CaCl_2$ was added (Thomas and Wolters, 1992; Stahl et al., 1995). Clearly, the efficacy of remediating water by adding salt is dependent on the source and chemical composition of the water.

The symptoms of reduced growth and feed conversion and/or death displayed by snapper in SG with low potassium concentrations are similar to those of chinook salmon fed potassium deficient diets (Shearer, 1988) and also for euryhaline species such as red drum, which were cultured in hypotonic water (Gatlin et al., 1992). In water with low ionic concentration, it is likely that there is a substantial loss of ions from the fish to the water. Osmoregulation, therefore requires increased expenditure of metabolic energy, which may

result in reduced fish performance (Bryan et al., 1988). We did not investigate the effects of low potassium concentration in SG on blood osmolality of snapper; however further research in this area is warranted.

Snapper grown in SG fortified with 40% of the potassium concentration as occurred in equivalent salinity CS continued feeding during the experiment and it is likely therefore that some potassium was being derived from the feed. We did not determine the potassium concentration in the feed, which was a high protein, fish meal-based diet. Fish meal is typically a poor source of potassium; however, soybean meal, which constituted 12% of the diet, contains in excess of 2% potassium (Lall, 1989). It is likely therefore that the diet contained at least 0.24% potassium. This diet concentration is similar to the potassium requirement level reported for the channel catfish, 0.26 % (Wilson and El Naggar, 1992) and some terrestrial animals such as rats, 0.23 % (Bieri, 1977) and pigs, 0.26% (Jensen et al., 1961). Clearly, the potassium concentration in the diet fed in our study did not provide adequate compensation for maximum growth of snapper grown in SG fortified with 40% potassium.

In general, dietary salt (NaCl) supplementation has resulted in little or no improvement in growth and feed conversion of some diadramous species such as Atlantic salmon grown in freshwater and seawater (Shaw et al., 1975), and rainbow trout, *Oncorhynchus mykiss* grown in freshwater (MacLeod, 1978). However, weight gain and food conversion of the euryhaline red drum grown in freshwater was improved when the diet was supplemented with NaCl at 2% or with both NaCl and KCl at 2% each (Gatlin et al., 1992). In our saline groundwater where potassium is deficient, improvements in performance of snapper may be achieved by supplementing the diet with KCl. This should be investigated.

It was not possible to acclimatise juvenile snapper to raw SG by gradually diluting SG fortified initially with either 60 or 100% of the potassium concentration as occurred in

equivalent salinity CS. Regardless of dilution schedule, all snapper became moribund or died when the potassium concentration in the SG (20‰) was diluted to 20% (~ 40 mg l^{-1}) of the potassium concentration as occurred in equivalent salinity CS. Clearly, snapper were unable to compensate for potassium concentration in the SG of approximately 40 mg l^{-1} or lower. There is very little information available on the physiological and morphological responses of teleosts, in particular marine-adapted fish, to low external ion concentrations. Perry and Wood (1985) showed that calcium uptake was higher in trout, *Salmo gairdneri* when they were held in water with low calcium concentration, compared to when they were held in water with high calcium concentration, and this was correlated with a proliferation of lamellar chloride cells. When Mozambique tilapia, *Oreochromis mossambicus* were held in magnesium deficient freshwater, magnesium deficiency in the fish coincided with increased calcium and sodium content and a low potassium content of the body. An increase in opercula chloride cell density was found also in magnesium deficient water (Bijvelds et al., 1997). Although there was no apparent difference in the performance of snapper grown in SG fortified with 60–100% of the potassium concentration as occurred in equivalent salinity CS, morphological and physiological changes may have occurred, particularly in response to lower potassium concentration in this range. Further research is warranted to determine if snapper compensate physically to SG that is partially deficient in potassium and also if different (e.g. longer) dilution schedules allow snapper to acclimatise to raw SG.

Our results suggest that for normal snapper performance and osmoregulation, SG (20‰) from Wakool must be fortified with potassium to provide a minimum concentration of approximately 120 mg l^{-1}. Because salinity of the groundwater can vary, and the interactions between ions during osmoregulation are complex, it may be more important to consider the ratio of ions rather than the specific concentration of individual ions in the

water. Forsberg et al. (1996) suggested that the survival of red drum grown in saline groundwater was correlated with K^+/Cl^- and Na^+/K^+ ratios; however, the correlations resulted from within-treatment variation where the highest (100%) and lowest (70%) survival occurred in replicates of the same groundwater treatment. Regardless of this, mean survival was high (85-100%) and instantaneous growth was the same for red drum grown in four different saline groundwaters, which were not fortified with potassium, suggesting that potassium was not deficient. In these authors' experiments, the K^+/Cl^- ratios of saline groundwater (15‰) and the artificial seawater control ranged from 0.007-0.014, and 0.022, respectively. In our study, the K^+/Cl^- ratios of SG treatment water ranged from 0.001 for raw SG to 0.018 for 100% fortified SG. Survival and growth of snapper was achieved in SG provided the K^+/Cl^- ratio was greater than 0.007, but maximum growth was achieved when the K^+/Cl^- ratio was greater than 0.01. When the K^+/Cl^- ratio was less than 0.007, snapper died.

5.6. Conclusion

Saline groundwater from Wakool was suitable for the culture of snapper in laboratory experiments provided the potassium concentration was fortified to supply a K^+/Cl^- ratio of 0.007-0.018. This was achieved easily by adding KCl to the saline groundwater. Following these results, a pilot-scale project has been established to evaluate the suitability of the saline groundwater for culture of snapper in ponds at Wakool. Further research should also investigate the potential to enhance growth of snapper in partially potassium deficient saline groundwater by adding potassium to the diet. Research should also be conducted to determine if snapper change morphologically and/or physiologically in response to suboptimal concentration of potassium in the saline groundwater.

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CHAPTER 6

The effects of changes in salinity on osmoregulation and chloride cell morphology of juvenile Australian snapper, *Pagrus auratus*

6. The effects of changes in salinity on osmoregulation and chloride cell morphology of juvenile Australian snapper, *Pagrus auratus*

6.1. Summary

The effect of rapid transfer of juvenile Australian snapper, *Pagrus auratus* from ambient seawater (30‰) to hyperosmotic (45‰) and near isoosmotic (15‰) environments on serum osmolality, serum $[Na^+]$,$[K^+]$, $[Cl^-]$, blood haematocit and branchial chloride cell morphology was assessed during 168 h after transfer. Serum osmolality, $[Na^+]$,$[K^+]$ and $[Cl^-]$ increased after 24 h in 45‰. In contrast, after 24 h in 15‰, $[K^+]$ did not change but serum osmolality, $[Na^+]$ and $[Cl^-]$ decreased. The serum chemistry changes were transient and had returned to near initial levels after 168 h in 45‰ and 15‰. Transfer from 30‰ to 45‰ and 15‰ did not affect blood haematocrit. Branchial chloride cells were identified in both filament and lamellar epithelia of snapper held in all salinity treatments by an immunocytochemical staining technique using an antiserum specific for Na^+, K+-ATPase. In 45‰, the number of filament and lamellar chloride cells did not change, but filament chloride cells were more abundant than lamellar chloride cells. In contrast, filament chloride cells had increased in size after 72 h and by 168 h after transfer from 30‰ were 1.4-fold larger than the initial size. In 15‰, the number of filament chloride cells and the size of both filament and lamellar chloride cells had decreased after 72 h. Our results demonstrate that snapper can osmoregulate in a wide range of salinity and suggest that both filament and lamellar chloride cells are responsible for excretion of excess salt from snapper in hyperosmotic environments.

6.2. Introduction

Many marine fish are able to osmoregulate and maintain ionic homeostasis in a wide range of salinities (Alderdice, 1988; Ferraris et al., 1988; Hwang et al., 1989; Munro et al., 1994; Jensen, et al. 1998; Uchida et al, 1996). Fish challenged with an altered environmental osmolality must maintain their body osmolality and ionic balance by changing behaviour, such as drinking rate (Tytler and Blaxter, 1988; Ura et al., 1996; Miyazaki et al., 1998), stress hormone levels, which can disturb hydromineral balance and blood parameters such as haematocrit (Woo and Chung, 1995; Wendelaar Bonga, 1997; Brown et al., 2001) and functions of the osmoregulatory surfaces (Hwang and Hirano, 1985; Hwang et al., 1989; Arai et al., 1997; Perry, 1998; Kelly and Woo, 1999). In metamorphosed fish, it is well established that branchial chloride cells, or "mitochondria-rich cells", are the primary site for discharge of excess Na^+ and Cl^- in an hyperosmotic environment (Zadunaisky, 1984; Alderdice, 1988) and uptake of Ca^{2+} and Cl^- in an hypoosmotic environment (Greco et al., 1995; Perry, 1998; Rombough, 1999). Na^+, K^+-ATPase is the primary driving force for flux of intra- and extracellular NaCl and is present in high concentrations on the basolateral side of chloride cells. Specifically it is localised in the tubular system membranes, which are extensions of the basolateral membranes (Hootman and Philpott, 1979; Karnaky, 1986; see review by Marshall and Bryson, 1998).

The number, size and location of branchial chloride cells are known to alter with changes in environmental salinity, however the degree of change in cells can be influenced by the range of change in salinity as well as the species and age of fish (Hwang and Hirano, 1985; Kelly and Woo, 1999; Kelly et al., 1999; Caberoy and Quinitio, 2000). Transfer of euryhaline fish from freshwater to saltwater generally causes a proliferation of branchial chloride cells and an increase in branchial Na^+, K^+-ATPase activity (Sasai et al., 1998). Two distinct types of chloride cells have been observed in filament and lamellar

epithelia of several species of diadromous and euryhaline teleosts using an immunocytochemical technique with an antiserum specific for Na^+, K^+,ATPase (Uchida and Kaneko, 1996; Uchida et al., 1996; Uchida et al., 1997; Sasai et al., 1998; Hirai et al., 1999). Changes in morphology of chloride cells following transfer to saltwater and freshwater suggest that filament and lamellar chloride cells are important in saltwater and freshwater osmoregulation, respectively.

Some species of sparids, or "sea bream", are euryhaline (Woo and Fung, 1981; Foscarini, 1988; Mancera et al., 1993; Kelly and Woo, 1999; Kelly et al., 1999); an attribute which has enhanced their suitability for aquaculture (Wu and Woo, 1983). Several studies on sparids have demonstrated that initial perturbations in osmolality and electrolyte concentration occurred when fish were transferred abruptly from seawater to hypoosmotic environments (6-10‰), however homeostasis was restored quickly (Woo and Fung, 1981, Mancera et al., 1993; Kelly and Woo, 1999; Kelly et al., 1999). Restoration of homeostasis was correlated with changes in the ultrastructure of chloride cells (Kelly and Woo, 1999; Kelly et al., 1999). On the other hand, few studies have been done on the effects of rapid transfer from seawater to hyperosmotic environments on changes in chloride cell morphology (Yoshikawa et al., 1993; Uchida et al., 2000).

Interest in aquaculture of the sparid, Australian snapper, *Pagrus auratus* is increasing (Battaglene and Fielder, 1997; Fielder et al., 2002, Chapter 2). This species is known to spawn in coastal and offshore conditions but juveniles usually inhabit estuarine environments (Fielder et al., 2001, Chapter 5), which typically experience daily and seasonal fluctuations in salinity. Preliminary studies on the effects of salinity on growth and survival of snapper larvae and juveniles have been done (Chapter 3), however the tolerance and osmoregulatory ability of snapper to salinity change has not been studied. The aim of the study was to assess the effects of rapid transfer of juvenile snapper from

ambient seawater (30‰) to low salinity (near-isoosmotic; 15‰) and high salinity (45‰) seawater on blood haematocrit, serum chemistry and branchial chloride cell morphology. The salinity treatments were selected to represent the salinity range that snapper are likely to encounter in Australian estuaries and aquaculture ponds.

6.3. Materials and methods

6.3.1. Source of juvenile snapper

Juvenile, first-generation hatchery-reared (G1) snapper were cultured at the Port Stephens Fisheries Centre (PSFC) using larval rearing techniques described in Fielder et al. (2001) (Chapter 5). Approximately 300 snapper were then on-grown using ambient estuarine water (range 30-35‰, 14-25°C) in a 10,000-l fibreglass tank (Tank 1). The water was filtered to 40 μm (Dega-Quiptron, Australia) and supplied constantly to the tank with a flow-through rate of approximately 1000 l h^{-1}. While in the tank, juvenile snapper were fed to satiation twice daily with a 45% protein, 1 to 4 mm pellet diet (Pivot, Australia).

6.3.2. Acclimation of fish prior to experiment

On 30 April 2001, ten fish were captured with hook and line from Tank 1 and blood samples were taken immediately using methods described in section 6.3.4. below, to provide baseline osmolality and serum ion concentration prior to disturbance of fish.

Tank 1 was then drained to 2500 l and snapper were anaesthetised with 20 mg l^{-1} of benzocaine. Three fish (245 ± 51 g; mean ± S.D., n = 81) were selected randomly and placed into each of twenty-seven, 400-l plastic-mesh cages, which were floating in three 10,000-l fibreglass acclimation tanks (three fish per cage and nine cages per tank). Each

acclimation tank was filled with sterilised 30‰ seawater (Fielder et al., 2002, Chapter 2) and operated on a common recirculation system with mechanical and biological filters and a water exchange of approximately 30 l min^{-1}. A black shade-cloth lid (~90% light reduction) was suspended over each experiment tank to reduce the intensity of ambient daylight. All tanks were housed in a polyethylene hot-house. Fish feeding protocols were the same as those described for Tank 1. Snapper remained in the acclimation tanks for seven days to avoid the potentially confounding effects of handling stress (e.g. increased production of blood cortisol) on osmoregulation.

After seven days, one cage was selected randomly in each acclimation tank. The three fish were removed and blood and gill samples were taken. This procedure was repeated sequentially for each remaining acclimation tank to provide data (n = 9 fish) for snapper immediately prior to transfer from 30‰ to treatment tanks (time 0). These samples were taken to confirm that initial handling and confinement in cages had not altered blood parameters and to provide data for initial gill chloride cell size and number.

6.3.3. Stocking and management of experiment tanks

Three 10,000-l fibreglass treatment tanks, situated in the same hot-house as the acclimation tanks, were selected randomly and filled with either 15‰, 30‰ or 45‰ water. The 15‰ and 45‰ water treatments were made by either diluting sterilised seawater (30‰) with fresh groundwater (0.6‰) or adding artificial sea salt (Instant Ocean®, Aquarium Systems Inc., USA), respectively. An acclimation tank was then randomly selected and the cage–held snapper were anaesthetised with 20 mg l^{-1} benzocaine. Each cage was then transferred from the acclimation tank to a single, randomly selected treatment tank (one cage per tank section; eight cages per treatment tank). This process was repeated for each of the remaining acclimation and treatment tanks at 20 minute

intervals, to ensure that all snapper were exposed to anaesthetic for the same length of time, and enable fish samples in individual treatment cages to be taken at the same time after transfer from 30‰. The experiment was conducted for seven days, during which time salinity in treatment tanks was maintained by adding fresh groundwater as required. Fish were not fed during the experiment. Disturbance to fish in adjacent treatment cages during times of sampling was reduced by black plastic curtains suspended from a central standpipe to the tank wall. Air was supplied to each tank section at approximately 1000 ml min^{-1}.

There is a potential shortcoming in the experimental design if tanks are considered the experimental unit, as tanks were not replicated. This was a result of either tank or saline groundwater availability. Instead, fish were considered as the experimental unit (n=6 fish) and protocols were incorporated to reduce the experimental error and to allow interpretation of the results with a high degree of certainty. These protocols included: fish for each sampling time were contained in cages within each treatment tank, and cages were separated from each other by black plastic partitions to reduce the degree of disturbance to adjacent cages.

6.3.4. Blood sampling

At 6 h, 24 h, 72 h and 168 h after transfer from 30‰ to treatment tanks, two cages from each treatment tank were selected randomly for sampling (n = 6 fish per treatment at each time). Three operators each removed a fish from the first cage using a hand-net and then restrained the fish in a plastic covered cavity which had been cut in a foam block. Within 2 min of capture, 0.5-1.0 ml of blood was taken from each fish by caudal puncture using 2 ml syringes and 25 gauge needles. Blood was transferred from syringes to Eppendorf tubes when a sub-sample of the blood was taken immediately by a 75 mm capillary tube for

estimation of haematocrit (Hct). After blood sampling, each snapper was placed into an individual aerated 60-l perspex tank filled with water from the respective treatment tank. Fish from the second cage were then blood sampled in the same way as those from the first cage. Blood was allowed to coagulate and was then stored on ice for up to 2 h. Serum was obtained by centrifugation at 6400 rpm for 2 min, then frozen and stored at $-18^{\circ}C$ until assay. A sample of water from each treatment tank was also taken at each time of sampling and stored frozen for osmolality and ion analyses (Table 6.1).

Table 6.1.

Mean osmolality and ion concentration of treatment water.

Treatment	Number of samples (n)	Osmolality (mmol kg^{-1})	$[Na^+]$ (mmol l^{-1})	$[Cl^-]$ (mmol l^{-1})	$[K^+]$ (mmol l^{-1})
Baseline (target 30‰)	2	881.0 ± 1.0	430.5 ± 3.5	498.0 ± 3.0	8.5 ± 0
Acclimatisation tanks (target 30‰)	3	905.3 ± 2.2	445.0 ± 2.1	515.0 ± 2.6	8.7 ± 0.03
15‰	4	435.8 ± 29.9	195.5 ± 15.0	225.5 ± 19.0	4.3 ± 0.2
30‰	4	905.8 ± 66.0	409.0 ± 36.8	492.3 ± 40.0	8.6 ± 0.6
45‰	4	1370.5 ± 22.2	605.5 ± 23.2	750.0 ± 39.8	13.5 ± 0.5

Haematocrit was measured after capillary tubes were centrifuged at 6400 rpm for 2 min. Serum and water osmolality were measured by freezing point depression (Advanced Micro-Osmometer, Model 3MO, Advanced Instruments Inc., USA). Serum and water $[Na^+]$, $[K^+]$ and $[Cl^-]$ were determined using ion-selective electrodes (Cobas Integra 700 ISE module, Roche Diagnostics, Switzerland). Osmolality and ion concentrations were analysed by a National Accredited Testing Agency, Hunter Area Pathology Service, Newcastle, Australia.

6.3.5. Immunocytochemical identification of chloride cells

Branchial chloride cells in gill filaments of treatment fish were detected by immunocytochemical staining techniques using an antiserum (NAK121) specific for Na^+, K^+ -ATPase (Ura et al., 1996). The specificity of the antiserum was confirmed in previous studies (e.g. Uchida and Kaneko,1996; Ura et al., 1996; Sasai et al., 1998; Hirai et al., 1999). After blood sampling at 0, 72 h and 168 h of the experiment, snapper were anaesthetised with 50 mg l^{-1} benzocaine until fish lost all equilibrium of buoyancy. They were weighed and then 10-20 pairs of gill filaments were removed from the left, second gill arch. Snapper were then revived by returning them to their respective 60-l perspex tank, which was supplied with compressed oxygen at approximately 1 l min^{-1}.

The gill filaments were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 h at 4°C and transferred to 70% ethanol and stored at 4°C. After fixation, the gill filaments were dehydrated in an ascending ethanol series of 90% ethanol for 1 h and 100% ethanol for 3 h (changed twice) and cleared in xylene for 2 h (changed once). Gill filaments were then embedded in paraffin. Serial sections (5 μm) were cut parallel to the long axis of the filament. Pairs of adjacent sections were mounted on separate silane-coated slides: one section was stained with anti-Na^+, K^+ -ATPase using techniques

modified from Sasai et al., (1998) and Hirai et al., (1999); the other section was stained with haematoxylin and eosin. In addition several sections were selected randomly and with all steps except the anti-Na^+, K^+-ATPase to allow confirmation of activity of the antiserum. The sections were immunocytochemically stained by the avidin-biotin-peroxidase complex (ABC) method (Hsu et al., 1981). The sections were deparaffined and then incubated sequentially at room temperature with (1) 3% H_2O_2 in methanol for 3 h to block endogenous peroxides in the gill tissue (2) 5% normal goat serum in 0.01 M phosphate buffered saline for 30 min to block non-specific staining sites (3) the primary antiserum, anti- Na^+, K^+ -ATPase diluted 1:6000 overnight (4) the biotinylated secondary antiserum, 0.5% biotinylated anti-rabbit IgG for 30 min (5) 0.2% Streptavidin (DAKO, Glostrup, Denmark) for 30 min (6) final visualisation step, Sigma Fast 3,3 diaminobenzidine Tablet Set (Sigma, Cat. No. D4293) for 5 min (7) stained with Mayer's haematoxylin for 10 seconds (8) stained with Scott's blue for 10 seconds (9) dehydrated in ascending ethanol series, cleared with xylene and coverslipped

The immunocytochemically stained sections and adjacent haematoxylin sections were then viewed using a light microscope (Olympus, Model BX41, Tokyo, Japan) and images were photographed using a digital camera (Olympus, Model C-4040Zoom, Tokyo, Japan) which was mounted on the microscope.

The number of chloride cells was determined for each fish by counting all strongly stained, immunoreactive cells on the afferent side of the filament and lamellae along a 500 μm length of filament. Sectional chloride cell size (μm^2) was estimated by measuring 20-30 cells, which were sectioned near the centre and included the nucleus using an image analyser (Scion Image for Windows, version Beta 4.0.2, Scion Corporation, MD, USA, http://www.scioncorp.com).

6.3.6. Daily water measurement

Salinity, temperature, pH and DO_2 were measured daily to the nearest 0.1‰, 0.1°C, 0.1 pH unit and 0.1 mg l^{-1}, respectively using a water quality meter (Horiba U-10, Horiba Ltd, Japan). Total ammonia ($\leq$ 0.4 mg l^{-1}) was measured daily with a rapid test kit (E. Merck, Model 1.08024, Germany).

6.3.7. Statistical analyses

Data were assessed for homogeneity of variance using the *F*- test. Means at each sampling time were compared with the initial mean (time 0) using Student's t-test. Unless otherwise stated, $\alpha = 0.05$. Statistical analyses were done using Statgraphics Plus 4.1(Manugistics, MD, USA).

6.4. Results

6.4.1. Serum osmolality, $[Na^+]$, $[K^+]$, $[Cl^-]$ and haematocrit

Blood haematocrit, serum osmolality and electrolyte concentrations did not differ between baseline concentrations of fish caught by hook and line, and fish acclimatised to experiment cages for 168 h (time 0), except for serum $[Cl^-]$ which was slightly but significantly higher in time 0 fish (Table 6.2).

Serum osmolality of fish maintained in 30‰ showed no significant changes but were slightly lower than the initial concentration during the experiment period of 168 h (Fig. 6.1). After direct transfer from 30‰ to 45‰, serum osmolality increased significantly by 24 h compared with the initial concentration, then decreased but by 168 h had returned to a concentration similar to the initial concentration (Fig. 6.1). In contrast, serum osmolality in

fish transferred directly from 30‰ to 15‰ decreased significantly by 6 h, remained lower than the initial concentration until 72 h, but increased to a concentration similar to the initial concentration by 168 h after transfer (Fig. 6.1).

Serum [Cl^-] of fish maintained in 30‰ was similar to the initial concentration during the 168 h experiment, except for a significant decrease at 72 h after transfer (Fig. 6.2). Serum [Cl^-] concentration of fish transferred from 30‰ to 45‰ increased by 24 h, then decreased below the initial concentration at 72 h and returned to near-initial concentrations by 168 h. Serum [Cl^-] of fish transferred from 30‰ to 15‰ decreased by 24 h, remained low to 72 h and although it then increased by 168 h it was still slightly but significantly lower than the initial concentration.

Serum [K^+] of fish maintained in 30‰ decreased significantly 6 h after transfer from the acclimation tank to the treatment tank, but [K^+] had increased to a concentration similar to the initial concentration by 24 h after transfer and remained constant at 72 h and 168 h sampling times (Fig. 6.3). Serum [K^+] of fish transferred from 30‰ to 45‰ increased after 24 h but then declined to reach a similar concentration to the initial level by 168 h. Serum [K^+] of fish transferred from 30‰ to 15‰ was not significantly different from the initial concentration at any sampling time (Fig. 6.3).

Serum [Na^+] of fish maintained in 30‰ remained constant during the experimental period of 168h, except for a small increase in concentration at 6 h after transfer (Fig. 6.4). Serum [Na^+] of fish transferred from 30‰ to 45‰ or 30‰ to 15‰ showed similar trends as that of serum [Cl^-] for fish transferred from 30‰ to 45‰. Serum [Na^+] increased by 24 h then it decreased by 72 h but by 168 h [Na^+] had returned to a concentration similar to the initial [Na^+]. For fish transferred from 30‰ to 15‰, [Na^+] decreased over 72 h, and by 168 h, although [Na^+] had increased, it was still slightly but significantly lower than the initial concentration (Fig. 6.4).

No significant difference in blood haematocrit of fish occurred in any treatments until 168 after transfer (Fig. 6.5). After this time, blood haematocrit was significantly lower than the initial level in fish transferred from 30‰ to 15‰ and 30‰. No fish died during the experiment.

6.4.2. Immunocytochemical detection of gill chloride cells

Immunoreactive chloride cells were distributed throughout the gill epithelia (Fig. 6.6). The number and size of round or columnar chloride cells located at the base of the lamellae and interlamellar regions (filament chloride cells) and flat chloride cells located on the lamellae (lamellar chloride cells) (Uchida et al., 1996; Hirai et al., 1999) varied when fish were transferred to different salinities (Fig. 6.6). Lamellar chloride cells were mostly located toward the distal end of the lamellae. Regardless of salinity treatment, filament chloride cells were more abundant than lamellar chloride cells (Fig. 6.6).

For fish transferred from 30‰ to 45‰, although the mean number of filament chloride cells remained the same as the initial number, the size of the cells increased significantly and were 1.4-fold larger than their initial size by 168 h (Fig. 6.7). For fish transferred from 30‰ to 45‰ there were no differences in the number and size of lamellar chloride cells compared with the initial values (Fig. 6.7).

For fish transferred from 30‰ to 15‰, the mean number of filament chloride cells decreased significantly by 72 h after transfer and by 168 h after transfer only 50% of the initial number of filament cells were found (Fig. 6.7). Similarly, filament chloride cells decreased significantly in size and were 35% smaller than the initial size by 168 h after transfer (Fig. 6.7). There was no difference between the number of lamellar chloride cells of fish transferred from 30‰ to 15‰ compared with the initial number (Fig. 6.7). However, the size of lamellar chloride cells of fish transferred from 30‰ to 15‰

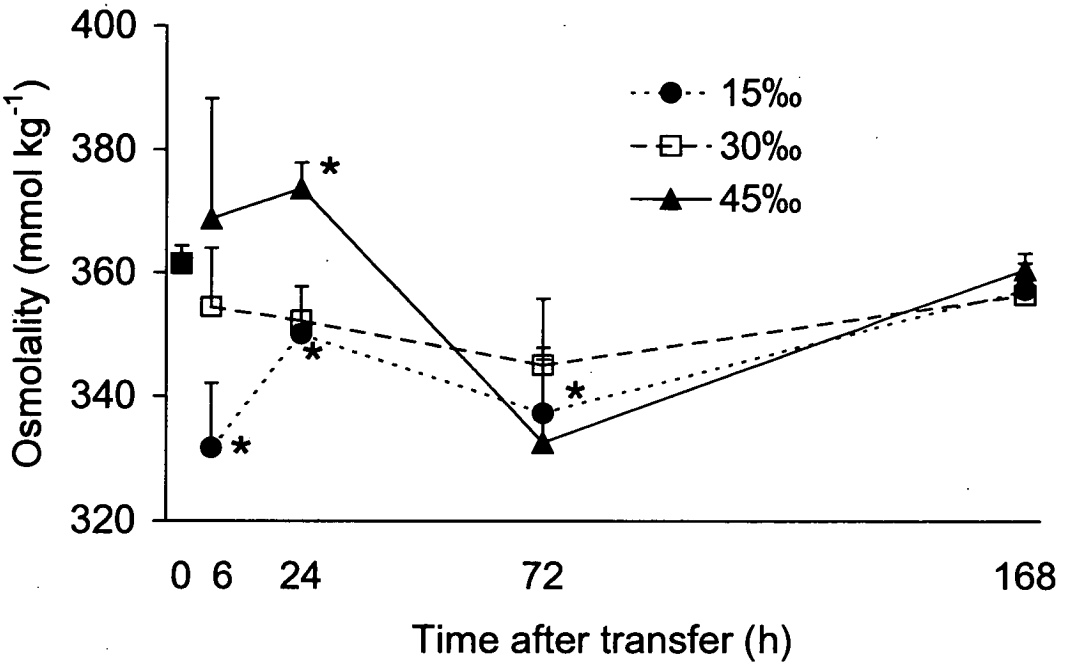
decreased significantly and cells were 29% smaller than the initial size after 168 h. (Fig. 6.7). Filament and lamellar chloride cell number and size, respectively did not change during the experiment in 30‰ control fish (Fig. 6.7).

Table 6.2.

Blood haematocrit, blood plasma osmolality and electrolyte concentration of juvenile snapper, *Pagrus auratus*, sampled by hook and line from a population of fish in a 10,000-l tank (baseline; n = 10) and after seven days acclimatisation in 100-l experiment cages (three fish per cage; n = 9) before fish were transferred from 30‰ to different salinities (time 0).

Treatment	Blood haematocrit (%)	Blood osmolality (mmol kg^{-1})	$[Na^+]$ (mmol l^{-1})	$[Cl^-]$ (mmol l^{-1})	$[K^+]$ (mmol l^{-1})
Baseline	51.5 ± 0.4^a	360.0 ± 2.9^a	176.5 ± 1.3^a	155.6 ± 1.3^a	6.8 ± 0.5^a
Time 0	48.7 ± 1.9^a	361.4 ± 2.9^a	176.4 ± 1.1^a	163.8 ± 1.2^b	6.3 ± 0.4^a

Data are means ± S.E.. Means within each column with a different superscript are significantly different ($P < 0.05$)



g. 6.1. Osmolality of blood serum of snapper transferred from 30‰ to 15‰ and 45‰. Points are means ± S.E. (n = 9 for time 0; n = 6 for other times). *, significantly different from the initial value ($P < 0.05$).

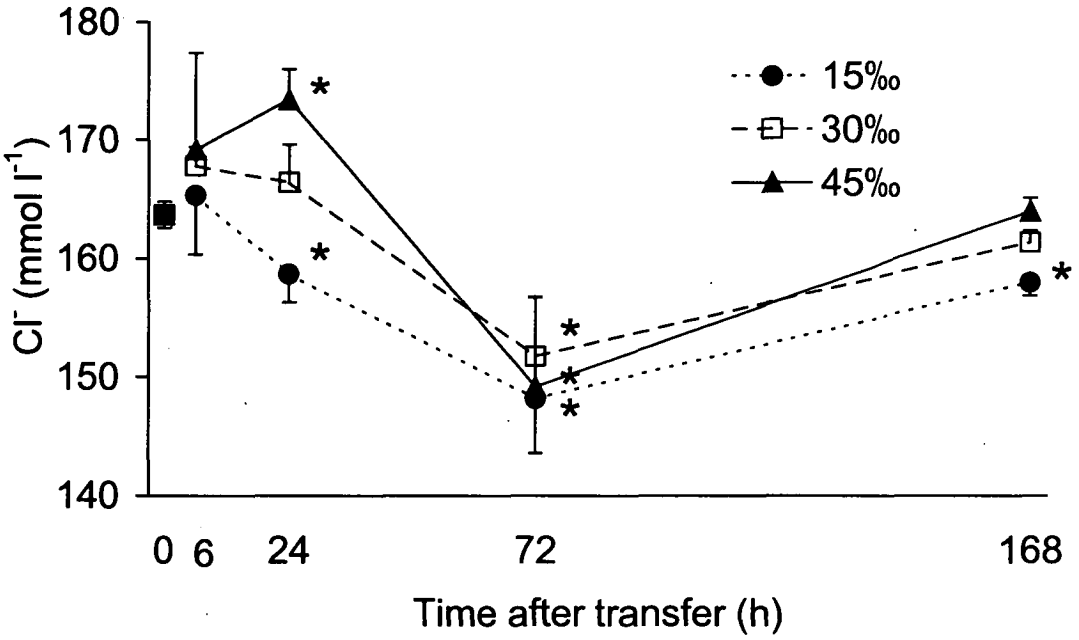


Fig. 6.2. Chloride concentration of blood serum of snapper transferred from 30‰ to 15‰ and 45‰. Points are means ± S.E. (n = 9 for time 0; n = 6 for other times). *, significantly different from the initial value ($P < 0.05$).

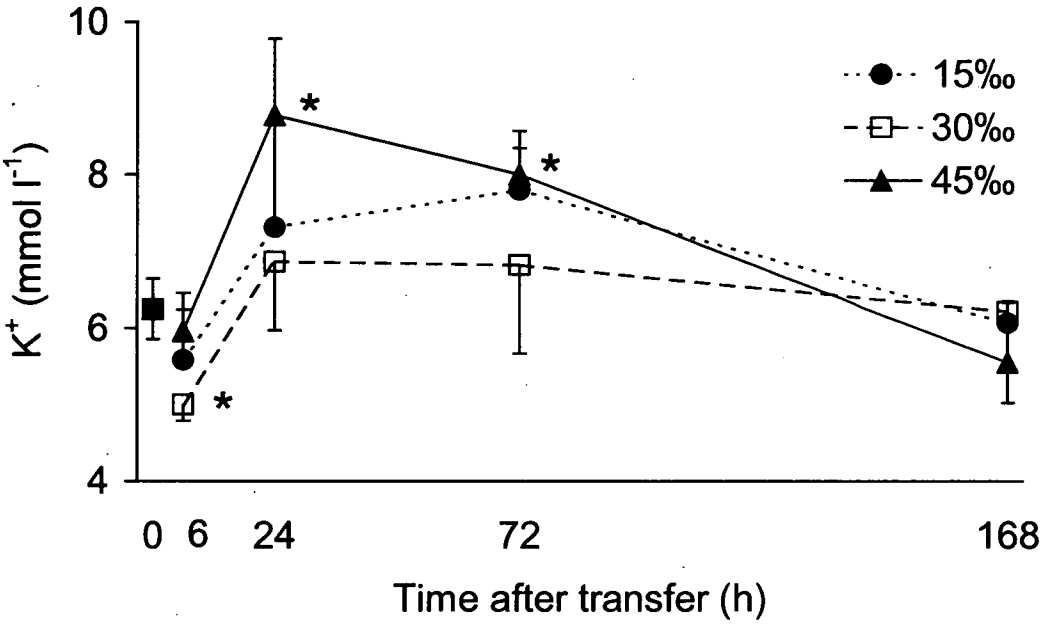


Fig. 6.3. Potassium concentration of blood serum of snapper transferred from 30‰ to 15‰ and 45‰. Points are means ± S.E. (n = 9 for time 0; n = 6 for other times). *, significantly different from the initial value ($P < 0.05$).

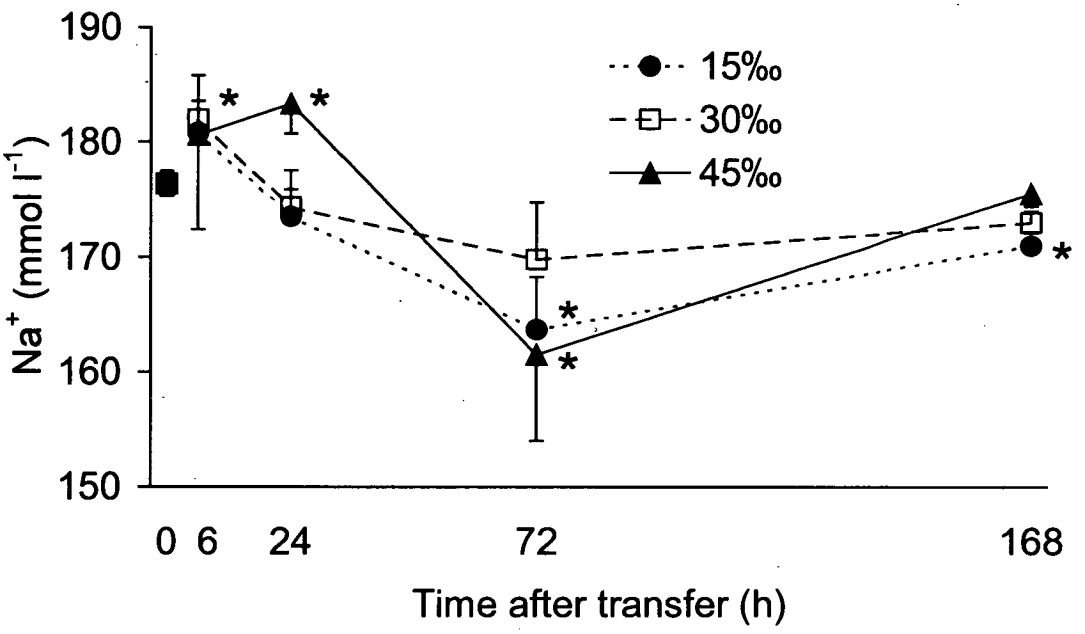


Fig. 6.4. Sodium concentration of blood serum of snapper transferred from 30‰ to 15‰ and 45‰. Points are means ± S.E. (n = 9 for time 0; n = 6 for other times). *, significantly different from the initial value ($P < 0.05$).

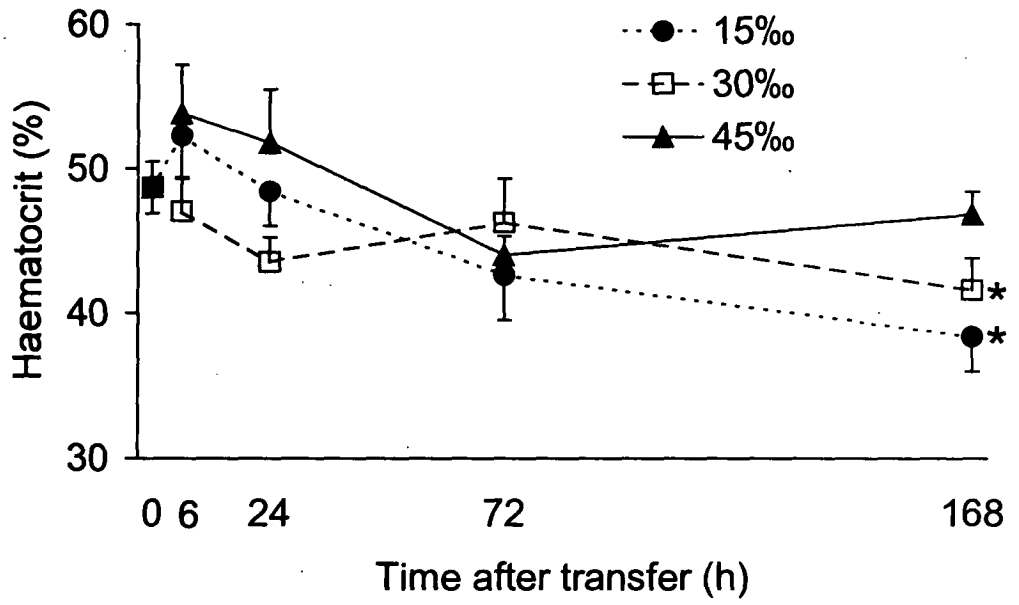
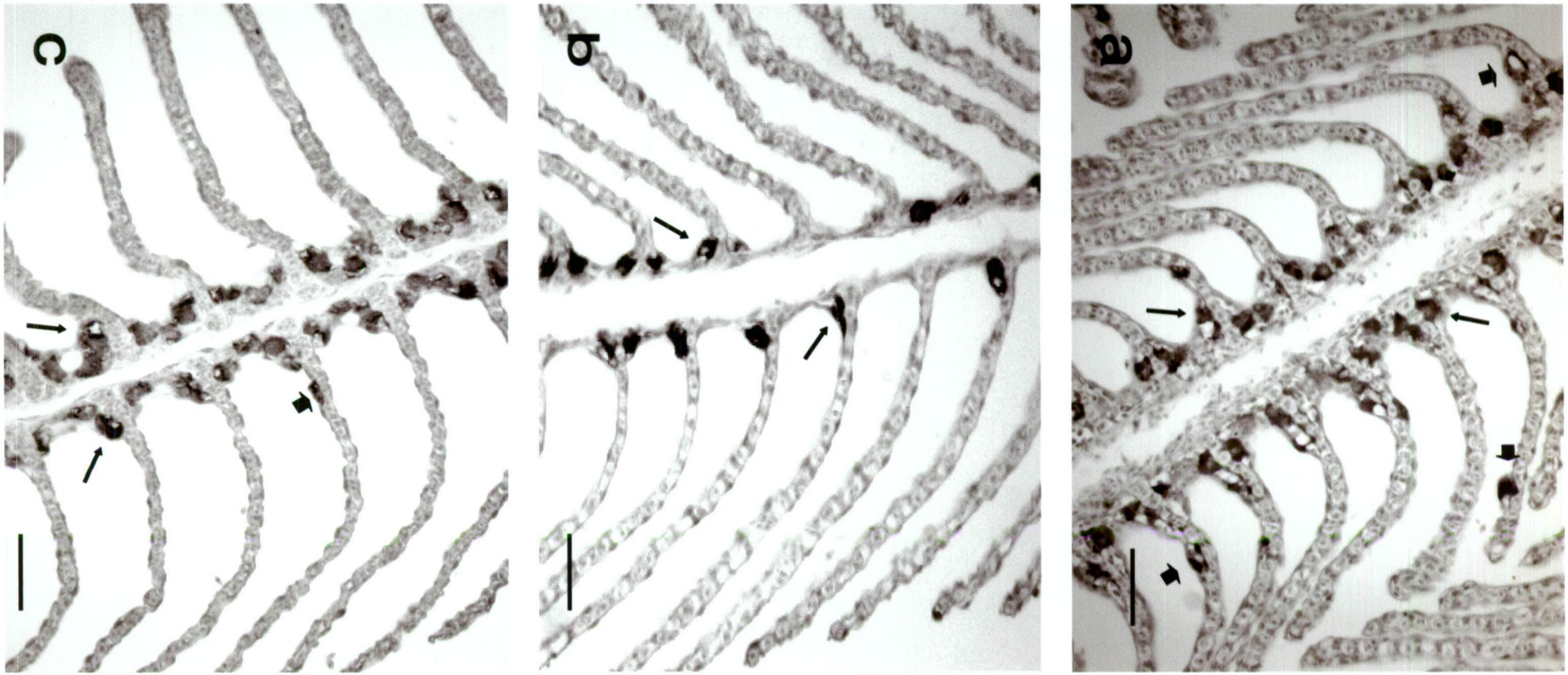


Fig. 6.5. Blood haematocrit (Hct) of snapper transferred from 30‰ to 15‰ and 45‰. Points are means ± S.E. (n = 9 for time 0; n = 6 for other times). *, significantly different from the initial value ($P < 0.05$).

Fig. 6.6. Sections of gills stained with an anti-Na^+,K^+-ATPase serum in juvenile snapper at 168 h after transfer from 30‰ to (a) 30‰, (b) 15‰ and (c) 45‰. Immunoreactive chloride cells are classified as filament (thin arrows) and lamellar (thick arrows) chloride cells according to their position and shape. Bar is 50μm



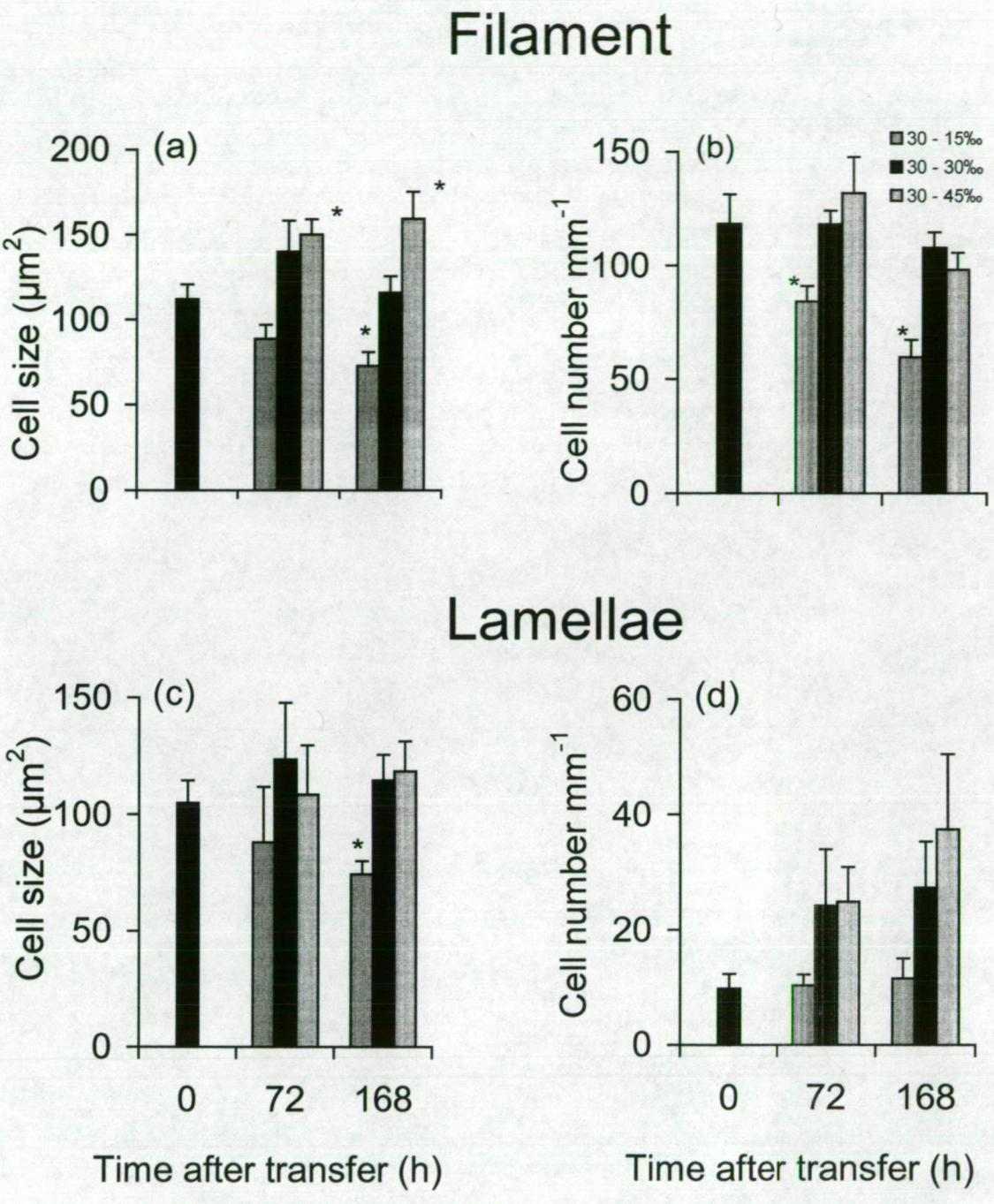


Fig. 6.7. Size and number of chloride cells in gill filaments (a, b) and lamellae (c, d) of snapper transferred from 30‰ to 15‰ and 45‰. Points are means ± S.E. (n=5). *, significantly different from the initial value ($P < 0.05$).

6.5. Discussion

Australian snapper tolerated direct exposure to concentrated, hyperosmotic (45‰, 1371 mmol kg^{-1}) and diluted, near-isoosmotic (15‰, 436 mmol kg^{-1}) environments following transfer from seawater (30‰, 906 mmol kg^{-1}). Serum osmolality and electrolyte concentration changed rapidly after transfer from 30‰, but the response differed according to osmolality of the external medium. Serum osmolality, $[Cl^-]$, $[Na^+]$ and $[K^+]$ of snapper increased in 45‰ after 24 h, indicating that initial dehydration occurred due to osmotic efflux of water from the fish and diffusional influx of electrolytes from the hyperosmotic environment (Bath and Eddy, 1979; Alderdice, 1988; Hwang et al, 1989). Conversely, although serum $[K^+]$ did not change significantly, serum osmolality, $[Cl^-]$, and $[Na^+]$ of fish transferred abruptly to 15‰ decreased initially, indicating that osmotic influx and diffusional efflux occurred with consequent dilution of serum electrolytes (Alderdice, 1988) or redistribution of ions between plasma and tissue cells (Bath and Eddy, 1979; Munro et al., 1994). These changes, however, were transient and after 72 h of exposure to 45‰ and 15‰, in general, the serum osmolality, $[Cl^-]$, $[Na^+]$ and $[K^+]$ started to approach the initial concentrations. By 168 h after transfer, blood osmolality and ion concentration had returned to initial levels.

Blood haematocrit was not affected by abrupt transfer from 30‰ to 45‰ and 15‰, however it was reduced slightly in fish held in 15‰ (38.4%) and 30‰ (41.6%, control) compared with the initial level (48.7%) after 168 h. The fact that blood haematocrit changed in control fish suggests that this change may have been influenced by a factor other than environmental osmolality. For example, haematocrit levels of snapper decreased following exposure to stress due to handling and confinement (Cleary, 1997). Although we attempted to maintain and handle all experiment fish in a low-stress environment (e.g. partitioned tanks, low light), as suggested by the fact that levels of haematocrit were the

same in undisturbed (baseline) and acclimatised fish before the start of the experiment, it is possible that an unknown stessor influenced haematocrit of some treatment fish at the 168 h sampling time. This is supported by Woo and Fung (1981) who showed an opposite effect of salinity on heamatocrit of red sea bream, *Pagrus major* (a sub species of snapper, *P. auratus*, Tabata and Taniguchi, 2000). In these fish haematocrit increased from 23.6% to 27.1% when fish were transferred from seawater (900 mmol kg^{-1}) to diluted seawater (470 mmol kg^{-1}). Regardless, haematocrit levels in our study were similar to the levels reported for snapper (Cleary, 1997) and within the range of 21% to 54% reported for red sea bream (Foscarini, 1988; Woo, 1990).

The reason for the general reduction in serum osmolality, $[Na^+]$ and $[Cl^-]$ in all treatments at 72 h after transfer is not clear, however stress associated with osmotic shock and/or confinement may also have contributed to this phenomenon. Stress hormones such as cortisol and catecholamine can be responsible for promoting active branchial extrusion of monovalent ions in marine species (Tort et al., 2001) as well as increasing rates of lamellar perfusion and passive movement of ions across branchial epithelia (McDonald and Milligan, 1997). Osmolality was correlated negatively with cortisol levels in gilthead sea bream, which were subjected to confinement and handling stress (Tort et al., 2001). Cleary (1997) showed that plasma cortisol levels of stressed snapper generally reached a maximum level 24 h after exposure to a handling stress but then declined to basal levels after 168 h. This author did not measure plasma cortisol after 72 h exposure to a handling stress, however cortisol may still be elevated at this time. Therefore it is possible that following the initial osmotic challenge, and associated immediate serum chemistry perturbations, and subsequent restoration of homeostasis, that stress hormones influenced plasma electrolyte flux.

Our results demonstrate that snapper can hypo-osmoregulate within a wide range of external salinity. This finding is consistent with those of other studies where the ability to osmoregulate over a range of environmental osmolalities was investigated for sparids such as red sea bream, (Woo and Fung, 1981), silver sea bream, *Sparus sarba* (Kelly and Woo, 1999), black sea bream, *Mylio macrocephalus* (Kelly et al., 1999), gilthead sea bream, *Sparus aurata* (Mancera et al., 1993) and other marine fish species (Ferraris et al., 1988; Jensen et al., 1998; Uchida et al., 2000; Foss et al., 2001).

The restoration of homeostasis in snapper transferred to a hyperosmotic and near isoosmotic environment was concomitant with rapid changes in the size and/or number of active branchial chloride cells. Filament and lamellar chloride cells have been identified using an immunocytochemical staining technique (similar to the one we used) in several species of freshwater-adapted and saltwater-adapted fish such as chum salmon, *Oncorhynchus keta* (Uchida et al., 1996), tilapia, *Oreochromis mossambicus* (Hiroi et al., 1998; Uchida et al., 2000), Japanese sea bass, *Lateolabrax japonicus* (Hirai et al., 1999) and Japanese eel, *Anguilla japonica* (Sasai et al., 1998).

Our results support the view that filament chloride cells are responsible for excretion of salt from fish in saltwater environments (Uchida and Kaneko, 1996; Uchida et al., 1997; Wales, 1997; Sasai et al., 1998; Hirai et al., 1999). Increased size of chloride cells in the hyperosmotic environment most likely reflects the generation of shallow leaky junctions and chloride cell complexes with subsequent increased ion permeability in response to the need for increased turnover rates of Na^+ and Cl^- (Hwang, 1990; Wales, 1997; Miyazaki et al., 1998)

Few studies have investigated the effects of rapid transfer of saltwater-adapted fish to hyperosmotic environments on changes in chloride cell morphology. Similar to our study, the number of branchial chloride cells in mudskipper, *Gillichthys mirabilis*, did not change,

but chloride cells became larger when fish were transferred from 30‰ to 60‰ (Yoshikawa et al., 1993). Also, filament chloride cells of tilapia acclimated to an hyperosmotic (~60‰) environment were two-fold and four-fold larger than those of fish held in seawater and freshwater, respectively (Uchida et al., 2000). In addition, chloride cells of these tilapia had well-developed tubular networks and an increase in the size of chloride cells was associated with a major elevation in Na^+, K^+-ATPase activity (Uchida et al., 2000). Jensen et al. (1998) also showed that gill Na^+, K^+-ATPase activity was increased significantly after 10 d when European sea bass, *Dicentrarchus labrax*, were transferred from 15‰ to 50‰ and 60‰, thus suggesting that commensurate changes in chloride cell morphology (increase in number or size) occurred. Indeed, branchial and/or opercular Na^+, K^+-ATPase activity has been shown to increase as salinity is increased in many fish species (McCormick, 1995). However, changes in chloride cell morphology and Na^+, K^+-ATPase activity in response to exposure to an hyperosmotic environment can be species-specific and may change during ontogeny. When 40 day old grouper, *Epinephelus coioides* were transferred from about 30‰ to 40‰ chloride cell number was increased but cell size was reduced, however when 60 day old juveniles were transferred from 30‰ to 60‰ the number and size of chloride cells and Na^+, K^+-ATPase activity did not change (Caberoy and Quinitio, 2000).

Similar, rapid responses in chloride cell morphology have also been observed when freshwater fish were transferred to seawater (Hwang and Hirano, 1985; Ayson et al., 1994; Uchida and Kaneko, 1996; Arai et al., 1997). For example, filament chloride cells of chum salmon fry increased significantly in size within 3 d of transfer from freshwater and were 1.8-fold larger than initial cells after 21 d. Lamellar chloride cells on the other hand, degenerated in seawater (Uchida et al., 1996). Interestingly, the number of lamellar

chloride cells in seawater-conditioned (~30‰) chum salmon fry and snapper in our experiment was similar at approximately 10 cells mm^{-1} of gill filament.

Lamellar chloride cells are often associated with adaptation to freshwater and are assumed to be responsible for ion uptake, especially Ca^{2+} and Cl^- (Laurent and Dunel, 1980; Perry and Laurent, 1989; Perry, 1997; Perry, 1998). Freshwater-adapted lamellar chloride cells are characterised by the absence of interdigitations and leaky-junctions between cells (Alderdice, 1988). Proliferation of lamellar chloride cells with concurrent degeneration of filament chloride cells following transfer from saltwater to freshwater has been observed in many fish species (Pisam et al., 1988; Uchida et al., 1997; Hirai et al, 1999). Snapper can survive in slightly hypoosmotic environments (~10‰) (Woo and Fung, 1981; Chapter 3), therefore the presence of lamellar chloride cells in seawater and hyperosmotic environments, albeit in low numbers, suggests that these fish may retain a degree of hypoosmotic adaptability. In the catadromous Japanese eel, lamellar chloride cells are present in similar densities in both freshwater-adapted and seawater-adapted fish (Sasai et al., 1998) and these authors suggest that the presence of both chloride cell types may allow a rapid osmoregulatory response to changes in external osmolality. In contrast, the euryhaline silver sea bream and black sea bream can hyper-osmoregulate in low environmental osmolality (6‰), but lamellar chloride cells were not present in fish held in seawater or 6‰ (Kelly et al., 1999; Kelly and Woo, 1999).

Lamellar chloride cells in snapper may also play a role in salt excretion. Lamellar chloride cells were observed in other marine fish, including yellowtail, *Seriola quinqueradiata* and frogfish, *Phrynelox tridens* however the density of lamellar chloride cells in the slow moving frogfish was much greater than that of the pelagic, fast-swimming yellowtail (Hughes and Umezawa, 1983; cited in Sala et al., 1987). Sala et al. (1987) made similar observations for the slow-moving skate, *Torpedo marmorata*, which has low

oxygen consumption in comparison with similar-size elasmobranchs. These authors suggested that the presence of lamellar chloride cells in marine fish might be related inversely to fish activity. Juvenile snapper are typically demersal feeders and are often found near seagrass beds and gravel/rocky substrate and are known to exhibit site fidelity to areas only a few hundred metres wide (Willis et al., 2001), suggesting a slow-moving habit. Further to this, the snapper used in our experiment were cultured in tanks with low turbulence and apart from twice daily periods of feeding activity, were relatively inactive. Our inactive, cultured snapper may have developed lamellar chloride cells.

A reduction in the size (filament and lamellar) and number (filament) of chloride cells in fish transferred from 30‰ to 15‰ likely reflects the reduced need for snapper to actively excrete Na^+ and Cl^- in the near-isoosmotic environment. Our observation of changing lamellar chloride cell morphology in response to a reduction in environmental salinity, as occurred with filament chloride cells, lends support to our hypothesis that lamellar chloride cells are involved in salt excretion in snapper. In contrast, the morphology of filament chloride cells and branchial Na^+, K^+-ATPase activity of black sea bream did not change when fish were grown in seawater (33‰) and an isoosmotic medium (12‰) for about 120 d (Kelly et al., 1999). Renal Na^+, K^+-ATPase activity however, decreased significantly in the 12‰ black sea bream suggesting that the need to exchange ions for urinary output was reduced in an isoosmotic environment (Kelly et al., 1999). Such differences within the same group of fish, sparids, confirms the view that the degree of morphological alteration of chloride cells in response to change in salinity is dependent on the range of salinity change in the environment and can vary with species of fish (Wu and Woo, 1983; Hwang and Hirano, 1985).

Although we did not examine ultrastructure of the chloride cells, a decrease in cell size in 15‰ probably indicates that some interdigitations and leaky junctions between chloride

cells had degenerated (Hwang and Hirano, 1985; Alderdice, 1988). Moreover, these changes probably started very quickly following transfer from 30‰ to 15‰. Hwang and Hirano (1985), using electron microscopy, observed similar changes in chloride cell morphology of juvenile flounder, *Kareius bicoloratus* within 3 h of transfer from 30‰ to 15‰. This trend continued for 3 d, however some chloride cells always retained the characteristics of seawater-adapted fish. Similarly, Kelly and Woo (1999) showed that changes in the ultrastructure of filament chloride cells of silver sea bream, such as evagination of the apical pit surfaces, occurred within 3 h of transfer from 33‰ to 6‰.

Notwithstanding the potential shortcoming in experimental design, tank effects do not appear an issue. This is evident by the fact that levels of blood serum osmolality and electrolytes were not different between baseline levels (fish angled from a free-swimming tank; 30‰; n=10) and control fish (n=9) which had been held for seven days in cages within a tank filled with seawater (30‰) (Table 6.2). This indicates that the tank had no effect on osmoregulation of snapper. Further, osmolality and serum electrolyte concentration of control fish were generally similar to baseline levels at all times of sampling. If there was a tank effect, perturbations in the parameters of control fish may be expected to occur. In contrast, significant perturbations in the serum parameters of treatment fish occurred, further suggesting that the responses were due to water treatment rather than to a tank effect.

Our results demonstrate that snapper can hypo-osmoregulate and maintain homeostasis in a wide range of salinities. This is an excellent attribute in support of their suitability for land-based aquaculture in ponds, where rapid fluctuation in salinity can occur due to evaporation or inundation with rain, and in several marine environments of South Australia (e.g. Spencers Gulf) and Western Australia (e.g. Shark Bay) where ambient salinity regularly exceeds 40‰. There is also significant interest in culturing snapper in inland

ponds designed for disposal of inland saline groundwater (Fielder et al., 2001, Chapter 5) and tolerance of a wide salinity range offers more opportunity to utilise saline groundwater from a number of sources, compared with a species that is relatively stenohaline.

6.6. Conclusion

Snapper tolerated abrupt exposure to hyperosmotic (45‰) and near-isoosmotic (15‰) environments. Serum osmolality, [Na^+] and [Cl^-] increased and decreased significantly within 24 h after transfer to 45‰ and 15‰, respectively. However, homeostasis was restored quickly in both environments and by 168 h after transfer, all measured parameters had returned to near-baseline levels. Restoration of homeostasis coincided with morphological changes in branchial chloride cells. Filament chloride cells increased in size in 45‰ and decreased in size in 15‰. Contrary to current views on chloride cell function, the presence of lamellar chloride cells in seawater-adapted snapper and their subsequent decrease in number and size in response to a reduction in environmental salinity suggests that these cells play a role in ion excretion.

6.7. Acknowledgements

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6.8. References

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CHAPTER 7

The effects of potassium concentration in saline groundwater on osmoregulation and chloride cell morphology of juvenile Australian snapper, *Pagrus auratus*

7. The effects of potassium concentration in saline groundwater on osmoregulation and chloride cell morphology of juvenile Australian snapper, *Pagrus auratus*

7.1. Summary

The effect of rapid transfer of juvenile Australian snapper, *Pagrus auratus* from coastal seawater (~30‰; CS) to saline groundwater (~30‰; SG), which was fortified with KCl to provide 4.5% (saline groundwater "raw"; SG-raw), 40% (SG-40K) and 100% (SG-100K) of the $[K^+]$ found in equivalent salinity seawater was assessed. Serum osmolality and $[Na^+]$, $[K^+]$, $[Cl^-]$, blood haematocrit and gill chloride cell morphology were measured for 168 h after transfer. Serum osmolality, $[Cl^-]$, $[Na^+]$ and $[K^+]$ in CS, SG-40K and SG-100K were generally similar to the initial levels, however osmolality and $[Cl^-]$ were elevated slightly in CS and SG-40K after 168 h. After 72 h of transfer from CS to SG-raw two fish had died and the remaining four fish were moribund. The serum osmolality, $[Cl^-]$ and $[Na^+]$ of fish transferred from CS to SG-raw increased rapidly and by 72 h were 7%, 7% and 3.3% higher than the initial levels, respectively. At the same time, serum $[K^+]$ of fish transferred from CS to SG-raw decreased and by 72 h was approximately 50% (1.5 ± 0.2 mmol kg^{-1}) of the initial level. Blood haematocrit of fish was not affected by water treatment. Branchial chloride cells were identified in both filament and lamellar epithelia of snapper in all water treatments by an immunocytochemical staining technique using an antiserum specific for Na^+,K^+-ATPase. The number and size of filament and lamellar chloride cells did not change when fish were transferred from CS to SG with different $[K^+]$. Filament chloride cells were more abundant than lamellar chloride cells and lamellar chloride cells were mostly located toward the distal end of the lamellae. Our results demonstrate that mortality of juvenile snapper following transfer from CS to SG-raw was due to the inability of fish to osmoregulate when the K^+/Cl^- ratio was 0.001. Provided the

K^+/Cl^- ratio in SG is 0.007 to 0.018 juvenile snapper can osmoregulate effectively. Branchial chloride cells did not change in response to low environmental $[K^+]$ suggesting that chloride cells in snapper are responsible for secretion of excess salt and not ion uptake, and that K^+ is obtained by snapper from other routes.

7.2. Introduction

Marine fish in seawater are hypoosmotic to the surrounding environment and tend to lose water by osmosis and gain Na^+ and Cl^- by diffusion. To compensate for the water loss, seawater-adapted teleosts drink seawater and then maintain ionic homeostasis of extracellular fluid by eliminating excess ingested ions. Most divalent ions remain in the gut and are evacuated from the anus while excess monovalent ions (Cl^-, Na^+) are actively secreted from the chloride cells of branchial surfaces (Alderdice, 1988; McCormick, 1995).

Chloride cells or "mitochondria-rich cells" are highly specialised and in seawater-adapted fish are characterised by an extensive tubular system which is continuous with the basolateral membrane. Na^+,K^+-ATPase, located in high concentrations on the basolateral membrane and tubular system, actively pumps Na^+ out of a chloride cell into the tubular system lumen and consequently creates a highly negative intracellular charge. Na^+ which enters the cell is recycled for K^+ via the Na^+,K^+-ATPase. The Na^+ gradient is then used to transport Cl^- into the cell from the blood through a secondary active $Na^+/K^+/2Cl^-$ cotransporter, which is also located in the tubular membrane. The Cl^- leaves the cell on an electrical gradient through an apical Cl^- channel. Na^+ is transported through a paracellular pathway down its concentration gradient (plasma being more positive than seawater) (see reviews by Pisam and Rambourg, 1991; McCormick, 1995). Both Na^+,K^+-ATPase and the $Na^+/K^+/2Cl^-$ cotransporter are dependent on K^+ and removal of K^+ from the basolateral side of chloride cells can rapidly inhibit the active transport of Cl^- (Marshall and Bryson, 1998).

Chloride and Na^+ are the dominant extracellular ions and K^+ is the main ion of intracellular fluids (Gardaire et al., 1991; Teeter, 1997). Although the $[K^+]$ of seawater is about 45-fold lower than that of $[Na^+]$ the seawater/plasma ratio of both ions is similar (~3:1) (Sanders and Kirschner, 1983a). Moreover, the total body content of K^+ is about 3-fold greater than Na^+ (Gardaire et al., 1991). Seawater fish obtain most of their K^+

requirement through ingestion of seawater and food (Sakamoto and Yone, 1978; Wilson and El Naggar, 1992; Lall, 1989) but K^+ can also be sequestered from the environment by diffusion across the branchial epithelium (Sanders and Kirschner, 1983b; Gardaire et al., 1991).

It is well known that chloride cell morphology of euryhaline fish can change following transfer from freshwater to seawater and vice versa (Laurent and Perry, 1991). Chloride cells in seawater-adapted fish are generally located on interlamellar regions of the gill filament and can increase in size and/or number as salinity is increased (Hwang and Hirano, 1985; Uchida and Kaneko, 1996; Uchida et al, 2000). In most cases, Na^+,K^+-ATPase activity also increases as salinity is increased (McCormick, 1995). In contrast, when fish are transferred from seawater to freshwater generally filament chloride cells degenerate and lamellar chloride cells proliferate, suggesting that the lamellar cells are involved with ion-uptake (see review by Perry, 1997). Both lamellar and to a lesser degree filament chloride cells of several species of fish have also proliferated following exposure to ion-poor freshwater (Laurent and Dunel, 1980; Perry and Laurent, 1989; Greco et al., 1995; Perry, 1997).

Unlike freshwater, which is highly variable in ionic composition, seawater is relatively stable in ionic composition and ratios of the major ions; Cl^-, Na^+, Mg^{2+}, Ca^{2+} and K^+ remain similar in estuaries regardless of salinity. Therefore osmoregulatory changes in estuarine fish are likely to be in response to altered environmental osmolality and total ion concentration rather than to changes in specific ion ratios. On the other hand, the chemical composition and ratios of major ions in saline groundwater (SG) can vary significantly from site to site and can differ from that of similar salinity coastal seawater (CS) (Forsberg et al., 1996; Fielder et al., 2001). Fielder et al. (2001) showed that the concentration of most of the major ions in SG from inland New South Wales, Australia was similar to CS.

However [K^+] of SG was approximately 4.5% of that found in similar salinity CS (K^+/Cl^- ratio 0.001). Bioassay and growth studies of the euryhaline sparid, Australian snapper, *Pagrus auratus* demonstrated that the SG taken directly from evaporation ponds was not suitable for survival and growth of snapper due to K^+ deficiency. However, it was possible to improve snapper performance by adding KCl to the SG. When SG was fortified to provide a K^+/Cl^- ratio of 0.007 snapper survived but did not grow as well as CS controls. However, when SG was fortified with K^+ to provide a K^+/Cl^- ratio of 0.01 to 0.018 snapper grew at similar rates as fish in CS. Fielder et al. (2001, Chapter 5) suggested that the differences in snapper performance in SG fortified with KCl were due to failure of fish to osmoregulate and maintain extracellular homeostasis.

A recent study showed that snapper have filament and lamellar chloride cells (Fielder et al., unpublished data). Both filament and lamellar chloride cells decreased in size when snapper were transferred abruptly from seawater to a near-isoosmotic environment. This suggests that lamellar chloride cells in snapper may play a role in salt excretion rather than ion uptake but this needs to be confirmed.

Several studies have investigated the effects of low environmental [K^+] in freshwater (Gardaire et al., 1991) and artificial seawater (Sanders and Kirschner, 1983b; Marshall and Bryson, 1998) on freshwater- and seawater-adapted fish or excised epithelial tissue to provide information on the kinetics and transport mechanisms of K^+ in chloride cells. However, to our knowledge, no studies have been done to determine the effects of low environmental K^+ in a saline environment on osmoregulation and chloride cell morphology of a euryhaline fish. The aim of the study was to assess the effects of rapid transfer of juvenile snapper from coastal seawater to saline groundwater, which was fortified with different K^+ concentrations (as a percentage of K^+ found in same salinity seawater) on

serum osmolality, serum concentration of Na^+, K^+ and Cl^-, blood haematocrit and gill chloride cell morphology.

7.3. Materials and methods

The experimental treatments (all 30‰) were:

(a) seawater to seawater (control, CS)

(b) seawater to 100% K^+-fortified saline groundwater (SG-100K)

(c) seawater to 40% K^+-fortified saline groundwater (SG-40K)

(d) seawater to raw (unfortified) saline groundwater (SG-raw)

7.3.1. Source of juvenile snapper

Juvenile, first-generation hatchery-reared snapper were cultured at the Port Stephens Fisheries Centre (PSFC) using larval rearing techniques described in Fielder et al. (2001, Chapter 2). Approximately 300 snapper were then on-grown in a 10,000-l fibreglass tank (Tank 1) until they were used in experiments. Ambient estuarine water (range 30-35‰, 14-25°C) was filtered to 40 µm (Dega-Quiptron, Australia) and supplied constantly to the tank with a flow-through rate of approximately 1000 l h^{-1}. While in the tank, juvenile snapper were fed to satiation twice daily with a 45% protein, 1 to 4 mm pellet diet (Pivot, Australia).

7.3.2. Source of saline groundwater and coastal seawater

Saline groundwater (SG; 66.2‰) was collected from an evaporation basin (Fielder et al., 2001, Chapter 5), transported in a fibreglass tank by road to PSFC, and stored in a

covered 3000-l tank. Coastal seawater (30‰) was filtered (1 μm) and stored in a 10,000-l fibreglass tank at PSFC.

7.3.3. Acclimation of experimental fish

On 23 August 2001, Tank 1 was drained to 2500 l and snapper were anaesthetised with 20 mg l^{-1} of benzocaine. Three fish (326 ± 76 g; mean ± S.D., n = 72) were selected randomly and placed into each of twenty, 200-l plastic-mesh cages, which were floating in a 10,000-l fibreglass acclimation tank. The acclimation tank was filled with filtered (1 μm) 30‰ seawater and operated on flow-through with a water exchange of approximately 15 l min^{-1}. Six airstones supplied air evenly through the tank. Each experimental cage had a black lid to reduce the intensity of ambient daylight. The acclimation tank was housed in an open-sided, covered shed. Fish were fed the same as that described for Tank 1. Snapper remained in the acclimation tank for seven days to avoid potential problems with osmoregulation due to handling and confinement stress.

7.3.4. Stocking and management of experimental tanks

Four 10,000-l epoxy-coated concrete experiment tanks, situated in the same shed as the acclimation tank, were filled with 3000 l of CS, SG-100K, SG-40K or SG-raw. The stock SG (66.2‰) was diluted with fresh groundwater to give 30‰ solutions (0.6 mmol l^{-1} K^+). The SG-100K and SG-40K treatments were then made by dissolving analytical grade KCl to give the required K^+ concentration (Table 7.1). No KCl was added to the SG-raw treatment water. Air was supplied by six evenly spaced airstones at approximately 1000 ml min^{-1} $airstone^{-1}$.

After seven days, two cages were selected randomly from the acclimation tank and the three fish from each cage were removed and blood and gill samples taken using methods described below to provide data (n = 6 fish) for snapper immediately prior to transfer from CS to SG treatment tanks (time 0).

Six randomly selected cages (each cage with 3 fish; total 18 fish) were transferred from the acclimation tank to each treatment tank (CS, SG-100K and SG-40K). Only four cages (12 fish) were transferred to the SG-raw treatment tank because previous research showed that snapper do not survive for more than 4 days in SG-raw water and it was expected that fish would not survive until the 168 h sample (Fielder et al., 2001, Chapter 5). Stocking times were staggered so that fish could be sampled after exactly the planned times in each treatment tanks. The experiment was conducted for seven days. Water exchange was minimal but salinity was maintained in treatment tanks by adding fresh groundwater (0.6‰) as required. Fish were not fed during the experiment.

7.3.5. Blood sampling

At exactly 24 h, 72 h and 168 h after transfer from CS to treatment tanks, two cages from each treatment tank were selected randomly for sampling (n = 6 fish per treatment at each time). Three operators each removed a fish from the first cage using a hand-net and then restrained the fish in a plastic covered cavity, which had been cut in a foam block. Within 2 min of capture, 0.5-1.0 ml of blood was taken by caudal puncture using non-heparinized syringes and 25 gauge needles. Blood was transferred from syringes to Eppendorf tubes and a sub-sample of the blood taken immediately by a 75 mm capillary tube for estimation of haematocrit (Hct). After blood sampling, each snapper was placed into an individual aerated 60-l perspex tank filled with water from its host treatment tank. Fish from the second cage were then blood sampled in the same way as those from the first

cage. Blood was allowed to coagulate and was then stored on ice for up to 2 h. Serum was obtained by centrifugation at 6400 rpm for 2 min, then frozen and stored at −18°C until assay. A sample of water from each treatment tank was also taken at each time of sampling and stored frozen for osmolality and ion analyses (Table 7.1).

Haematocrit was measured after capillary tubes were centrifuged at 6400 rpm for 2 min. Serum and water osmolality were measured by freezing point depression (Advanced Micro-Osmometer, Model 3MO, Advanced Instruments Inc., USA). Serum and water $[Na^+]$, $[K^+]$ and $[Cl^-]$ were determined using ion-selective electrodes (Cobas Integra 700 ISE module, Roche Diagnostics, Switzerland). Osmolality and ion concentrations were analysed by a National Accredited Testing Agency, Hunter Area Pathology Service, Newcastle, Australia.

7.3.6. Immunocytochemical identification of chloride cells

Branchial chloride cells in gill filaments of treatment fish were detected by immunocytochemical staining techniques using an antiserum (NAK121) specific for Na^+, K^+ -ATPase (Ura et al., 1996). The specificity of the antiserum was confirmed in previous studies (e.g. Ura et al., 1996; Uchida and Kaneko,1996; Sasai et al., 1998; Hirai et al., 1999). After blood sampling at 0, 72 h (fish from SG-raw) and 168 h (CS, SG-100K and SG-40K) snapper were anaesthetised with 50 mg l^{-1} benzocaine until fish lost all equilibrium of buoyancy. They were weighed and then 10-20 pairs of gill filaments were removed from the left, second gill arch. Snapper were then revived by returning them to their host 60-l perspex tanks, which were supplied with compressed oxygen at approximately 1 l min^{-1}.

The gill filaments were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 h at 4°C and transferred to 70% ethanol and stored at 4°C. After fixation, the

gill filaments were dehydrated in an ascending ethanol series of 90% ethanol for 1 h and 100% ethanol for 3 h (changed twice) and cleared in xylene for 2 h (changed once). Gill filaments were then embedded in paraffin. Serial sections (5 μm) were cut parallel to the long axis of the filament. Pairs of adjacent sections were mounted on separate silane-coated slides: one section was stained with anti-Na^+, K^+ -ATPase using techniques modified from Sasai et al., (1998) and Hirai et al., (1999); the other section was stained with haematoxylin and eosin. The sections were immunocytochemically stained by the avidin-biotin-peroxidase complex (ABC) method (Hsu et al., 1981). The sections were deparaffined and then incubated sequentially at room temperature with (1) 3% H_2O_2 in methanol for 3 h to block endogenous peroxides in the gill tissue (2) 5% normal goat serum in 0.01 M phosphate buffered saline for 30 min to block non-specific staining sites (3) washed with phosphate buffered saline for 10 min (4) the primary antiserum, anti- Na^+, K^+ -ATPase diluted 1:6000 overnight (5) the biotinylated secondary antiserum, 0.5% biotinylated anti-rabbit IgG for 30 min (6) 0.2% Streptavidin (DAKO, Glostrup, Denmark) for 30 min (7) final visualisation step, Sigma Fast 3,3 diaminobenzidine Tablet Set (Sigma, Cat. No. D4293) for 5 min. (8) stained with Mayer's haematoxylin for 10 seconds (9) stained with Scott's blue for 10 seconds (10) dehydrated in ascending ethanol series, cleared with xylene and coverslipped

The immunocytochemically stained sections and adjacent haematoxylin sections (to confirm immuno stained cells) were then viewed using a light microscope (Olympus, Model BX41, Tokyo, Japan) and images were photographed using a digital camera (Olympus, Model C-4040Zoom, Tokyo, Japan) which was mounted on the microscope.

The number of chloride cells was determined for each fish by counting all strongly stained, immunoreactive cells on the afferent side of the filament and lamellae along a 500 μm length of filament. Sectional chloride cell size (μm^2) was estimated by measuring 20-

30 cells, which were sectioned near the centre and included the nucleus using an image analyser (Scion Image for Windows, version Beta 4.0.2, Scion Corporation, MD, USA, http://www.scioncorp.com).

7.3.7. Daily water measurement

Salinity, temperature, pH and DO_2 were measured daily in each treatment tank using a water quality meter (Horiba U-10, Horiba Ltd, Japan). Total ammonia (≤ 1.0 mg l^{-1}) was measured daily with a rapid test kit (E. Merck, Model 1.08024, Germany).

7.3.8. Statistical analyses

Data were assessed for homogeneity of variance using the *F*- test. Means at each sampling time were compared with the initial mean (time 0) using Student's t-test. Unless otherwise stated, $\alpha = 0.05$. Statistical analyses were done using Statgraphics Plus 4.1(Manugistics, MD, USA).

7.4. Results

7.4.1. Serum osmolality, $[Na^+]$, $[K^+]$, $[Cl^-]$ and haematocrit

After 72 h of transfer from CS to SG-raw two fish had died and the remaining four fish were moribund. Water temperature in all treatment tanks was similar and within the range of 17.3-24.5°C until 144 h after transfer from CS. However, after this time a heater in the CS and SG-40K treatments malfunctioned overnight and at the 168 h sampling time the water temperature had increased to about 26.0°C (Table 7.1).

Serum osmolality of fish increased in all treatments after 24 h, but by 72 h after transfer serum osmolality had returned to near initial levels in CS, SG-100K and SG-40K (Fig. 7.1). In contrast, serum osmolality of fish transferred to SG-raw continued to increase and was almost 7% higher than the initial level after 72 h. After 168 h, serum osmolality in SG-100K had not changed but was significantly higher in CS and SG-40K than the initial level.

Serum [Cl^-] of fish in all treatments was not different from the initial concentration 24 h after transfer from CS, however by 72 h after transfer [Cl^-] was slightly but significantly elevated in CS and SG-100K fish and was 7% higher than the initial concentration in SG-raw fish. Serum [Cl^-] in fish in SG-100K was similar to initial levels but was slightly higher in CS and SG-40K at 168 h after transfer from CS (Fig. 7.2).

Serum [K^+] of fish transferred from CS to CS, SG-100K and SG 40 was similar in general to the initial concentration at all sample times. The serum [K^+] in SG-raw tended to decrease with time but [K^+] was not significantly different from the initial value after 24 h or 72 h of transfer from CS (Fig. 7.3).

Serum [Na^+] of fish transferred from CS to CS, SG-100K and SG-40K was similar to the initial concentration at all sampling times, with the exception of SG-100K after 168 h, which was slightly but significantly lower than the initial value. In contrast, the serum [Na^+] of fish transferred from CS to SG-raw increased rapidly after 24 h and by 72 h after transfer was significantly higher (3.3%) than the initial concentration (Fig. 7.4). Blood haematocrit of fish was not affected by water treatment (Fig. 7.5).

7.4.2. Immunocytochemical detection of gill chloride cells

Immunoreactive chloride cells were distributed throughout the gill epithelia. The number and size of round or columnar chloride cells located at the base of the lamellae and

interlamellar regions (filament chloride cells) and flat chloride cells located on the lamellae (lamellar chloride cells) (Uchida et al., 1996; Hirai et al., 1999) did not changewhen fish were transferred from coastal seawater to groundwater with different [K^+] (Fig. 7.6). Filament chloride cells were more abundant than lamellar chloride cells (Fig.7.6). Lamellar chloride cells were mostly located toward the distal end of the lamellae.

7.5. Discussion

Serum osmolalities and [Cl^-], [Na^+] and [K^+] of fish transferred from coastal seawater (CS) to SG fortified with K^+ to provide 40% (3.5-3.9 mmol kg^{-1}) and 100% (8.7-9.4 mmol kg^{-1}) of that found in equivalent salinity seawater (~30-35‰; 877-970 mmol kg^{-1}) were generally similar to the initial levels during 168 h. However, minor increases in serum osmolality and [Cl^-] had occurred by 168 h in CS and SG-40K treatments. It is possible that a rapid increase in water temperature from approximately 21°C to 26°C due to malfunction of immersion heaters in these treatment tanks and a commensurate increase in salinity due to evaporation after 144 h caused the hydromineral changes. The ability to osmoregulate for any given fish species tends to be greatest at near-optimum or lower temperatures and decreases in supra-normal temperatures (Kinne, 1963). The optimal temperature for juvenile snapper osmoregulation is not known however premetamorphosis snapper larvae performed best in terms of growth, development and survival at 24°C (Chapter 3). Therefore 26°C may be supra-optimal for osmoregulation in juvenile snapper. Osmotic permeability of the plasma membrane increases with temperature (Alderdice, 1988) and stress associated with rapid changes in temperature and salinity can cause increases in passive ion influxes and water loss, and inhibit active ion exchange in seawater-adapted fish (Wendelaar Bonga, 1997). Also, gill Na^+,K^+-ATPase activity generally decreases following acclimation to high temperature (McCormick, 1995). These

factors coupled with increased environmental salinity may have led to reduced secretion of excess Cl^-.

The ability for Australian snapper to maintain ionic homeostasis in raw saline groundwater (SG-raw) was affected profoundly by the external $[K^+]$. Fish transferred from CS to unfortified SG (K^+, 0.6 mmol kg^{-1}; SG-raw) were unable to maintain ionic homeostasis. Serum osmolality and $[Cl^-]$ and $[Na^+]$ had increased but serum $[K^+]$ had decreased by about 50% of the initial level (1.5 ± 0.2 mmol kg^{-1}) after 72 h and fish had either died or were moribund. This indicates the effects of low environmental K^+ on reducing blood $[K^+]$ and the subsequent failure of the mechanisms in chloride cells to secrete Cl^- and Na^+ from the blood.

Increased serum $[Cl^-]$ and $[Na^+]$ may have occurred directly as a result of failure of the $Na^+/K^+/2Cl^-$ cotransporter, which is dependent on K^+, and/or indirectly by blockade of Na^+,K^+-ATPase and subsequent reduction in the transmembrane Na^+ gradient which drives the cotransport (see review by Marshall and Bryson, 1998). The former effect however may have had the greater influence on snapper osmoregulation. Marshall and Bryson (1998) demonstrated in the seawater-adapted killifish, *Fundulus heteroclitis* that complete removal of K^+ from a saline solution on the basolateral side of opercular epithelial tissue, which contains chloride cells with the same ultrastructure as branchial cells (Foskett et al., 1981; Zadunaisky, 1984), inhibited rapidly the secretion of Cl^- from cells. In contrast, inhibition of the Na^+,K^+-ATPase pathway by adding ouabain to a saline

Table 7.1.

Ranges of water quality parameters during the experiment for the acclimation tank (AT), coastal seawater (CS) and saline groundwater treatments (SG)

Treatment	DO_2 (mg l^{-1})	pH	Temperature (°C)	Salinity[c] (‰)	Osmolality[c] (mmol kg^{-1})	Na^+ (mmol l^{-1})	K^+ (mmol l^{-1})	Cl^- (mmol l^{-1})	K^+/Cl^- ratio
AT	7.2-8.8	7.8-8.4	16.2-18.2	31.3-33.7	944	433	9.1	519	0.018
CS	6.4-8.2	8.1-8.4	17.3-26.1	32.8-37.4	970-1091	445-499	9.3-10.3	528-591	0.018
SG-100K[a]	7.0-7.8	8.1-8.4	20.5-24.5	30.4-34.1	893-970	338-362	8.7-9.4	522-561	0.017
SG-40K[b]	6.3-8.7	8.2-8.4	18.9-26.0	30.0-34.6	877-989	333-373	3.5-3.9	510-570	0.007
SG-raw	7.3-8.5	7.7-8.3	17.6-18.5	30.2-30.4	865-876	336	0.6	507-519	0.001

[a]Saline groundwater fortified with KCl to target 100% of K^+ as found in same salinity coastal seawater

[b]Saline groundwater fortified with KCl to target 40% of K^+ as found in same salinity coastal seawater

[c]range was due to evaporation from treatment tanks during the experiment

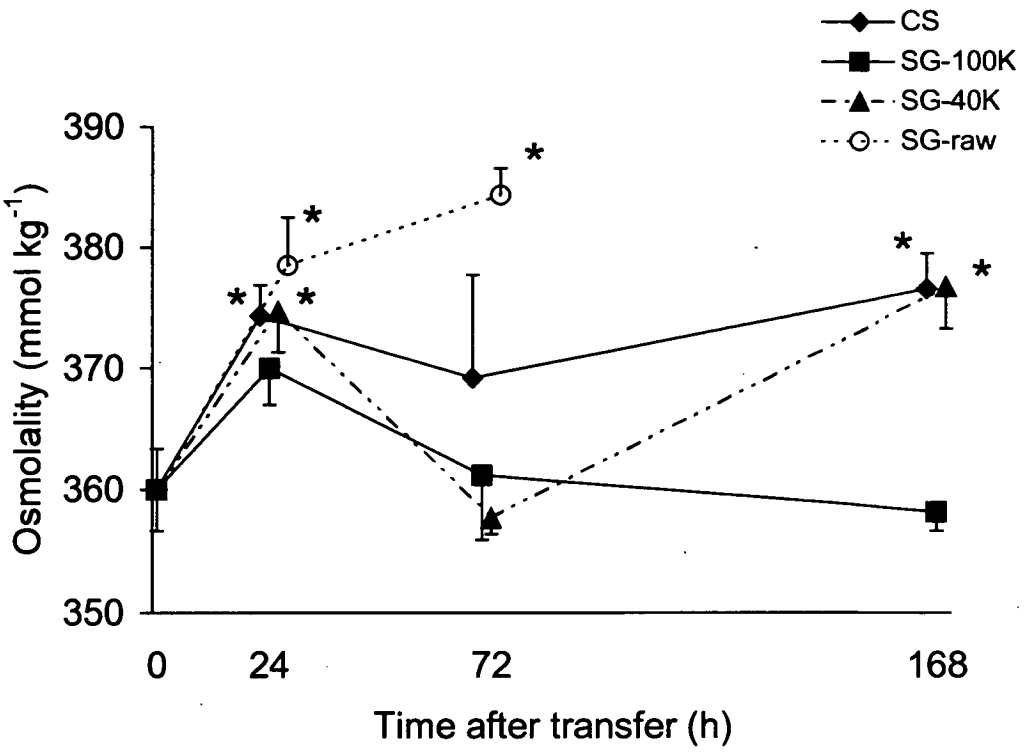

Fig. 7.1. Osmolality of blood serum of snapper transferred from coastal seawater (CS) to saline groundwater fortified with KCl to provide 4.5% (SG-raw), 40% (SG-40K) and 100% (SG-100K) of $[K^+]$ found in same salinity seawater. Points are means ± S.E. (n=4 for SG-raw; n=6 for others). *, significantly different from the initial value (P < 0.05).

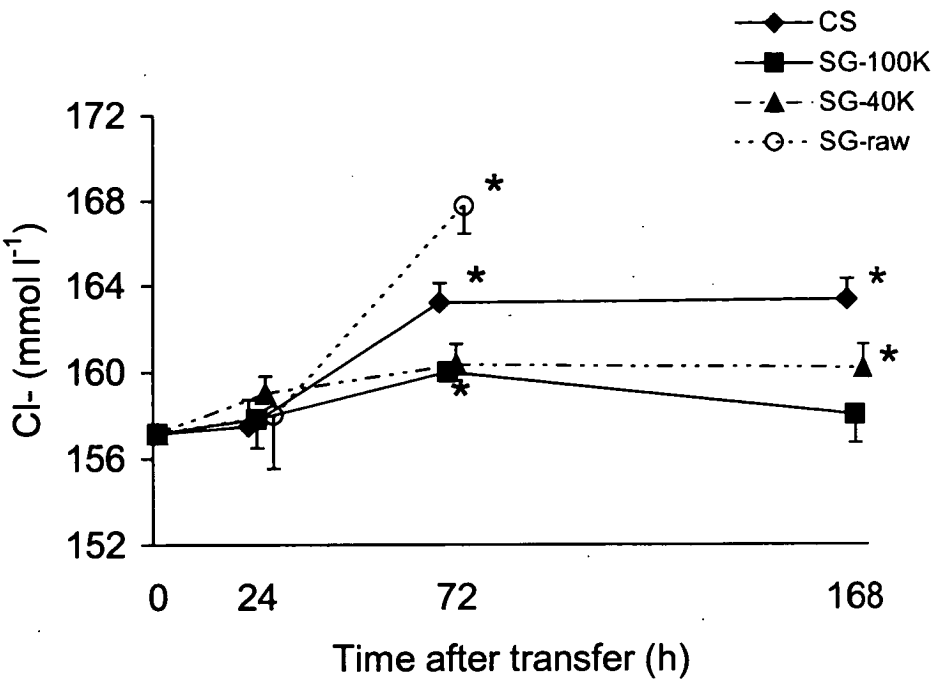

Fig. 7.2. Chloride concentration of blood serum of snapper transferred from coastal seawater (CS) to saline groundwater fortified with KCl to provide 4.5% (SG-raw), 40% (SG-40K) and 100% (SG-100K) of [K^+] found in same salinity seawater. Points are means ± S.E. (n=4 for SG-raw; n=6 for others). *, significantly different from the initial value ($P < 0.05$).

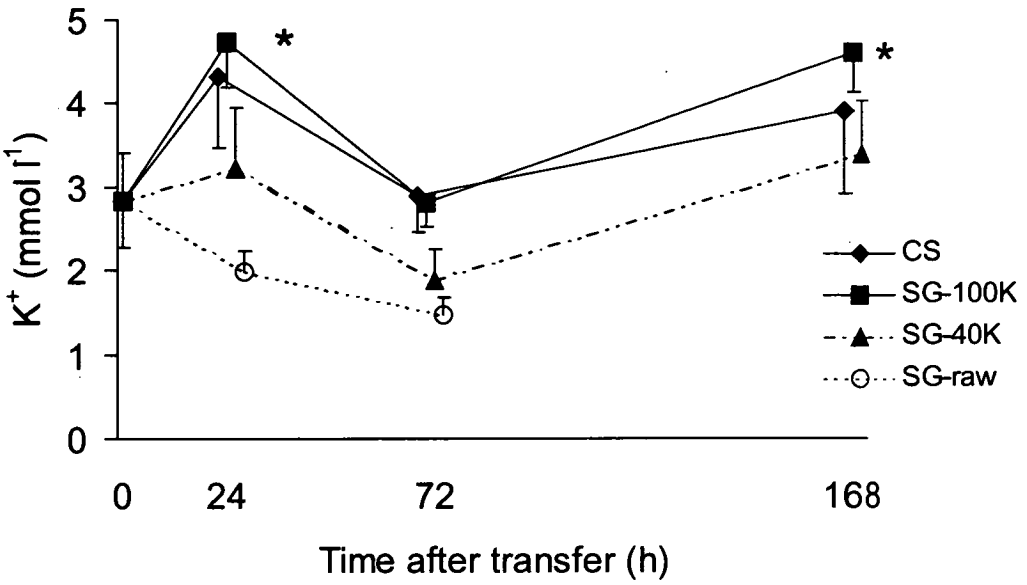

Fig. 7.3. Potassium concentration of blood serum of snapper transferred from coastal seawater (CS) to saline groundwater fortified with KCl to provide 4.5% (SG-raw), 40% (SG-40K) and 100% (SG-100K) of [K^+] found in same salinity seawater. Points are means ± S.E. (n=4 for SG-raw; n=6 for others). *, significantly different from the initial value ($P < 0.05$).

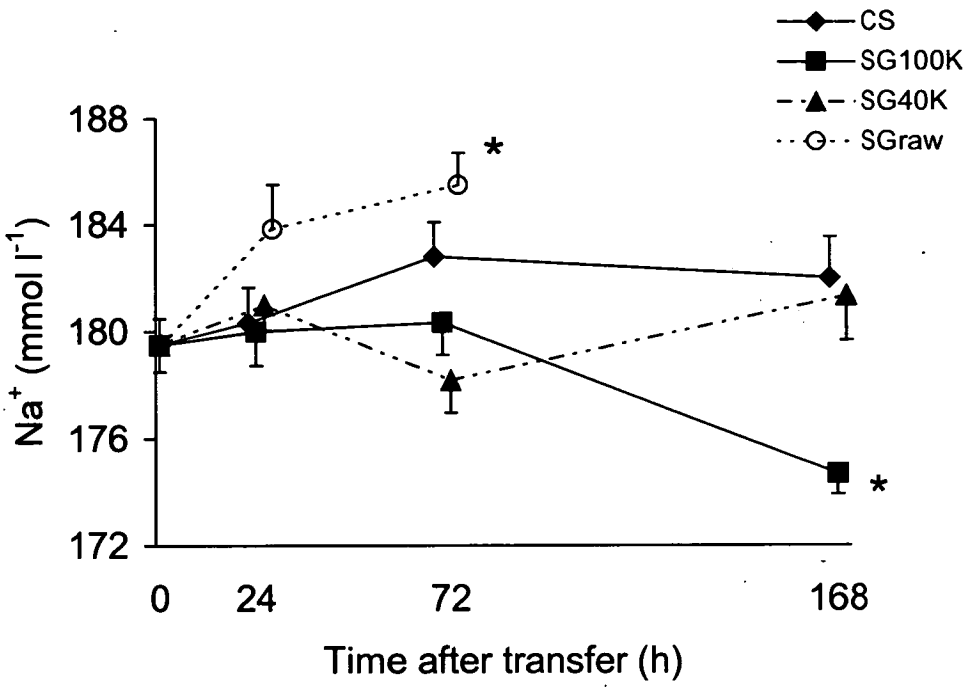

Fig. 7.4. Sodium concentration of blood serum of snapper transferred from coastal seawater (CS) to saline groundwater fortified with KCl to provide 4.5% (SG-raw), 40% (SG-40K) and 100% (SG-100K) of [K^+] found in same salinity seawater. Points are means ± S.E. (n=4 for SG-raw; n=6 for others). *, significantly different from the initial value ($P < 0.05$).

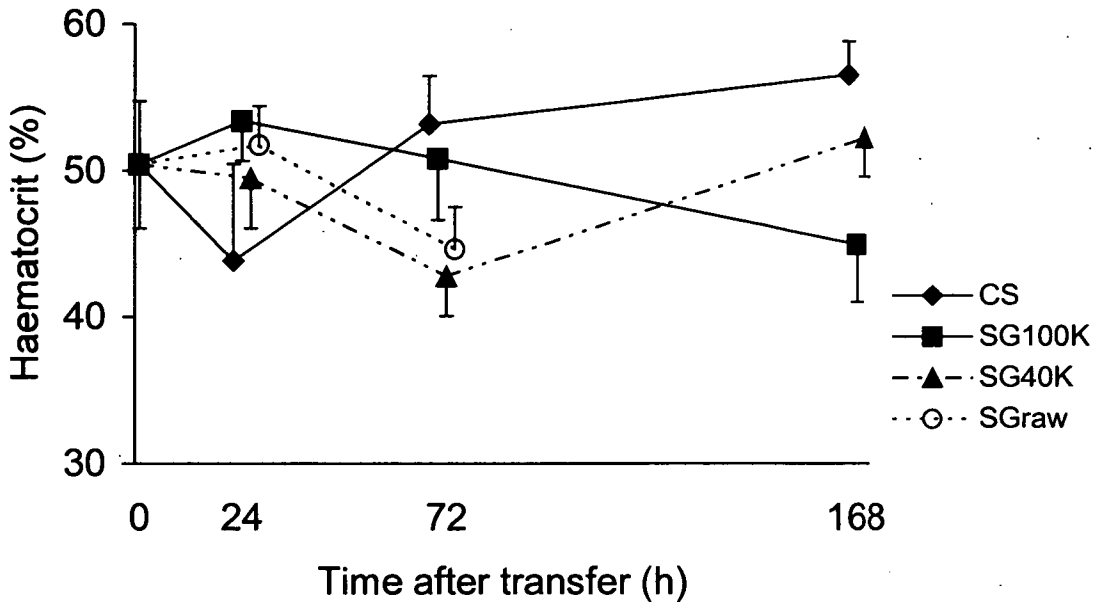

Fig. 7.5. Blood haematocrit (Hct) of snapper transferred from coastal seawater (CS) to saline groundwater fortified with KCl to provide 4.5% (SG-raw), 40% (SG-40K) and 100% (SG-100K) of $[K^+]$ found in same salinity seawater. Points are means ± S.E. (n=4 for SG-raw; n=6 for others). *, significantly different from the initial value (P < 0.05).

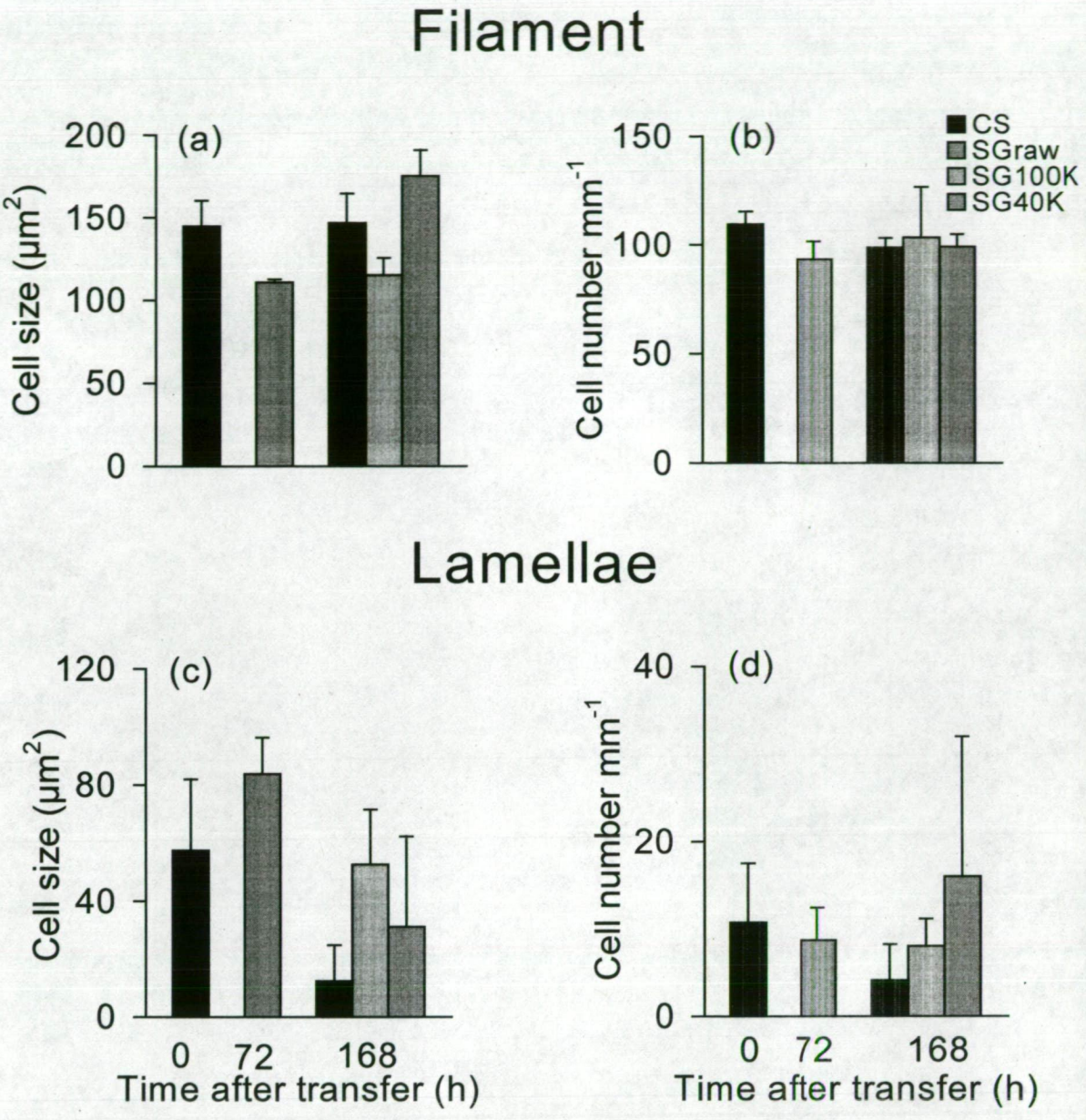

Fig. 7.6. Size and number of branchial filament (a, b) and lamellar (c, d) chloride cells in snapper transferred from coastal seawater (CS) to saline groundwater fortified with KCl to provide 4.5% (SG-raw), 40% (SG-40K) and 100% (SG-100K) of $[K^+]$ found in same salinity seawater. Points are means ± S.E. (n=4 for SG-raw; n=6 for others). *, significantly different from the initial value ($P < 0.05$).

solution elicited a much slower reduction in secretion of Cl^-. The activity of Na^+,K^+-ATPase in gill tissue of European sea bass, *Dicentrarchus labrax* was also decreased significantly when environmental [K^+] was excluded from saline water; the optimal ion concentrations for Na^+,K^+-ATPase activity were 54 mmol l^{-1} Na^+ and 15 mmol l^{-1} K^+ (with 2.5 mmol l^{-1} Mg^{2+}) (Jensen et al., 1998). Exchange of other essential ions may also be disturbed as a consequence of Na^+,K^+-ATPase blockage. For example, ouabain-induced reduction in Cl^- secretion rates of killifish inhibited apical uptake of Ca^{2+} (Marshall and Bryson, 1998).

Excessive loss of intracellular K^+ may have also contributed to death of snapper in SG-raw. The apical membrane of branchial chloride cells is permeable to Na^+ and K^+ and there is evidence to suggest that K^+ efflux from seawater teleosts is partly passive diffusion and partly active transport while branchial influx of K^+ is through passive diffusion (Sanders and Kirschner, 1983b). The active extrusion process of K^+ is not strongly activated by environmental [K^+], however it is sensitive to [Na^+] or osmotic pressure of the environmental fluid (Sanders and Kirschner, 1983b). These authors showed that efflux of K^+ from seawater-adapted trout, *Salmo gairdneri* and sculpin, *Leptocottus armatus* did not change when environmental [K^+] of seawater was altered (0 – 30 mmol kg^{-1}) and that efflux of K^+ was about 3 times greater than influx of K^+. This resulted in a net loss of K^+ from the fish when environmental [K^+] was 0 and 10 mmol kg^{-1}. Changes in plasma [K^+] were augmented by transfer of intracellular K^+ rather than by diffusional influx of K^+ from the environment. Although the [K^+] in SG-raw was 6-fold less than that in SG-40K and 16-fold less than that in SG-100K and CS (~10 mmol kg^{-1}) the osmolality and [Na^+] in all of our treatments were similar suggesting that K^+ efflux remained similar regardless of environmental [K^+]. The permeability of the basolateral membrane of chloride cells to K^+ is high (Gardaire et al, 1991) and there is a large degree of K^+ recycling across this

membrane (Marshall and Bryson, 1998). In snapper held in SG-raw, it is therefore possible that in response to deficient blood [K^+] excessive mobilisation (dilution) of intracellular K^+ contributed to cell dysfunction and fish death.

Blood haematocrit was not affected by SG treatment. This suggests that osmotic flux did not vary after fish were transferred from CS to treatment solutions of similar osmolality (Bath and Eddy, 1979; Alderdice, 1988), [Cl^-] and [Na^+] and further supports our observation that change in serum ion concentration of fish transferred to SG-raw was due to low [K^+] of the water.

Branchial chloride cells were identified in both filament and lamellar epithelia of snapper, however no significant change in cell size or number was observed in any treatment during 168 h after transfer from CS. Filament chloride cells are responsible for excretion of excess salt from fish in saltwater environments and often increase or decrease their size and number when environmental salinity is increased or decreased, respectively (Foskett et al., 1981; Zadunaisky, 1984; review by Pisam and Rambough, 1991). Lamellar chloride cells on the other hand are mostly associated with adaptation to freshwater and are assumed to be responsible for active ion uptake, particularly Ca^{2+}, Cl^- and Na^+ (review by Perry, 1997) and also K^+ (Gardaire et al., 1991). Proliferation of lamellar chloride cells and concurrent degeneration of filament chloride cells have been observed in many fish species following transfer from seawater to freshwater (Laurent and Dunel, 1980; Pisam et al., 1990; Sasai et al., 1998; Hirai et al., 1999). Also, lamellar chloride cells proliferated in freshwater-adapted fish after exposure to artificially softened freshwater (Greco et al., 1995) or freshwater which was deficient in Ca^{2+} or Mg^{2+} (Laurent and Perry, 1991; Bijvelds et al., 1997; Perry, 1998). However, lamellar chloride cells of snapper were observed recently to decrease in size following transfer from seawater (30‰) to near-

isoosmotic (15‰) environments, suggesting that these cells play a role in salt excretion and not ion uptake (Fielder et al., unpublished data).

In our experiment, despite different [K^+], the CS and SG treatments had similar salinity (30-33‰), [Cl^-] and [Na^+] and consequently were hyperosmotic to snapper. Therefore to maintain homeostasis snapper needed to drink saline water and secrete excess ions, particularly Cl^- and Na^+ from the chloride cells (Kirsch et al., 1984; McCormick, 1995). The fact that lamellar chloride cells did not increase in size or number in SG with low environmental [K^+] when the need to sequester K^+ was increased, supports the theory that snapper lamellar chloride cells are involved with salt excretion and also that the principal route of K^+ absorption in saltwater-adapted snapper is via the gut from ingested seawater (Sakamoto and Yone, 1978).

Slight differences in initial serum [K^+] and initial lamellar chloride cell size occurred between experiments in Chapter 6 (6.2 mmol l^{-1} [K^+]; 100 μm^2) and Chapter 7 (3.5 mmol l^{-1} [K^+]; 60 μm^2; this chapter). The reasons for these differences are unclear, however it is possible that the initial serum [K^+] in Chapter 6 was elevated due to lysis of red blood cells and subsequent release of intracellular K^+ during handling of whole blood (e.g. blood extraction, centrifuging). A different group of fish was used for each experiment. Although rearing conditions were similar for each group prior to their use in an experiment, it is possible that the activity of fish in each group was different with subsequent differences in the initial lamellar chloride cell size between groups (see Chapter 6).

Survival and performance of some fish when transferred from freshwater to seawater or vice versa has been improved by acclimation at an intermediate salinity (Hwang, 1987). The step-wise transfer reduced the degree of osmotic challenge following each transfer and enabled commensurate morphological changes in chloride cells to occur while homeostasis was maintained. This is unlikely to occur for snapper in our SG as previous attempts failed

to acclimate juvenile snapper from SG collected from the same source as this experiment (Fielder et al., 2001). These authors, initially fortified SG with 100% of K^+ found in same salinity CS (20‰) and then diluted $[K^+]$ slowly over 42 days. When $[K^+]$ was 20% or less ($K^+/Cl^- < 0.007$) than that found in seawater, snapper died. This result further supports our observation that snapper are unable to osmoregulate in saline water with low K^+/Cl^- ratio and that chloride cell morphology does not change in response to exposure to low environmental $[K^+]$.

Although a close relative of snapper, the red sea bream, *Chrysophrys major* obtained all essential K^+ from seawater (Sakamoto and Yone, 1978), significant quantities (more than 50%) of the essential K^+ influx for marine fish can be ingested with food (Sanders and Kirschner, 1983b). We did not feed our snapper during the 168 h experiment period therefore feeding, especially with feeds fortified with K^+, may have enhanced the ability for snapper to osmoregulate in K^+-deficient saline groundwater (Fielder et al., 2001, Chapter 5). This should be investigated.

Our results confirm that mortality of juvenile snapper following transfer from CS to SG-raw was due to the inability of fish to osmoregulate when the K^+/Cl^- ratio was 0.001. Provided the K^+/Cl^- ratio in SG is ≥ 0.007 juvenile snapper can osmoregulate effectively. However the energy cost of osmoregulation is not known. Fielder et al. (2001, Chapter 5) showed that growth and feed intake of snapper was lower in SG with a K^+/Cl^- ratio of 0.007 compared with SG with a K^+/Cl^- ratio of 0.01-0.018. These authors suggested that the energy cost to maintain homeostasis in SG with low $[K^+]$ was greater and therefore growth was lower than in SG with high $[K^+]$. Stress can also manifest as lower fish growth and lower feed intake by reallocating metabolic energy away from growth and reproduction towards hydromineral regulation (Wendelaar Bonga, 1997). Fish in SG with a

K^+/Cl^- ratio of 0.007 may therefore be chronically stressed and further research is warranted to determine changes in levels of stress hormones with external $[K^+]$ in SG.

7.6. Acknowledgements

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CHAPTER 8

General Discussion

8. General Discussion

8.1. Introduction

The viability of existing snapper, *Pagrus auratus* farms and future development of the industry in Australia is dependent on a reliable supply of cheap, high quality fingerlings and identification of new land-based growout sites, especially in inland areas with saline groundwater (Chapter 1).

The aims of my thesis were to identify the optimal physical regime for intensive rearing of snapper larvae, to evaluate the suitability of saline groundwater for culture of snapper and to determine the ability of snapper to osmoregulate in coastal seawater and inland saline groundwater from New South Wales.

Larval rearing trials were done with snapper larvae, which were spawned from captive, first-generation broodstock following hormone induction or environmental manipulation. Larvae were reared intensively through to metamorphosis using live feeds and laboratory experiments were done to determine optimal photoperiod, salinity and temperature. Results of laboratory experiments were evaluated/validated in a commercial-scale tank trial.

Saline groundwater and osmoregulation trials were done with tank-reared juvenile snapper in laboratory experiments using coastal seawater, and saline groundwater collected from an evaporation pond in inland New South Wales.

8.2. Larval rearing

Optimising development, growth and survival of larvae in hatcheries will reduce the cost of fingerlings by reducing the number of non-viable, malformed larvae (e.g. uninflated swimbladder; spinal and jaw deformities) and allowing more, larger batches of fingerlings to be produced each year. Successful intensive larval rearing of marine fish larvae requires

a high degree of knowledge of specific environmental and biotic needs of larvae (Howell et al., 1998; Shields, 2001). Photoperiod, salinity and temperature alone, or in combination can have profound effects on the development, growth and survival of marine fish larvae (e.g. Kinne, 1963; Battaglene, 1995; Rombough, 1996; Hart et al., 1996; van der Kraak and Pankhurst, 1996; Howell et al., 1998; Pankhurst and Hilder, 1998; Boeuf and Le Bail, 1999; Cox and Pankhurst, 2000).

Teleost larvae must pass through a series of critical periods to continue development and failure to do so can impact significantly on the success of hatchery production. Blaxter (1988) suggested that there are five critical larval periods: hatching, first-feeding, respiration, swimbladder inflation and metamorphosis. Successful passage of larvae through each of these critical periods can be influenced by either one or all of the photoperiod, salinity and temperature parameters (Blaxter, 1988). The optimal conditions for each of these physical parameters can differ between species and may change during larval ontogeny as a critical developmental period may be influenced more or less by one or a combination of parameters (Barnabé, 1990; Ronzani Cerqueira and Chatain, 1991; Battaglene and Talbot, 1990, 1992; Barlow et al., 1995; Hart et al., 1996; Rombough, 1996; Shields, 2001; Chapters 2 and 3). In addition, ambient combinations of these abiotic parameters normally experienced by larvae in the wild may not necessarily be optimal for intensive larval rearing (Tandler et al., 1989; Tandler et al., 1995). Therefore it is essential to determine the optimal physical parameters for larval rearing in replicated laboratory experiments.

Conditions which encourage maximum growth of early stage larvae may not provide the best environment for first-feeding, swimbladder inflation and survival (Battaglene and Talbot, 1990; Ronzani Cerqueira and Chatain, 1991; Chapter 2). Long photoperiod can increase the success of first-feeding, growth and development to the detriment of

successful swimbladder inflation (Ronzani Cerqueira and Chatain, 1991; Battaglene, 1995; Parra and Yúfera, 2000; Chapter 2). Successful swimbladder inflation is also reduced at the upper or lower extremities of the tolerable range for temperature or salinity in snapper (Chapter 3) and gilthead sea bream (Tandler et al., 1995). However, once these developmental milestones have been achieved conditions for maximum growth are desirable to ensure larvae reach metamorphosis and can be weaned from live feeds onto pellet diets and transferred from the hatchery to nursery systems. Most marine fish larvae must reach a certain size before they can metamorphose into a juvenile fish (Youson, 1988). Length and/or growth rate of fish larvae are two of the most important factors that determine development and the onset of metamorphosis (Youson, 1988).

Post-swimbladder inflated snapper larvae grew best at an intermediate, extended photoperiod (18 h light : 06 h dark); longer than under the near-ambient light period necessary for optimal swimbladder inflation but shorter than under continual light. Similar observations have been made with growth of European sea bass (Barahona-Fernandes, 1979) and most likely reflect the energy budget of periods of activity which use energy (feeding during the light phase) and periods of inactivity which conserve energy (dark phase) (Kitajima et al., 1985; Kitajima et al., 1993; Blaxter, 1986; Pankhurst et al., 1991; Chapter 2). As in the European sea bass, snapper may also have a circadian feeding pattern and do not feed for periods of the day (0000 h to 0800 h) regardless of the presence of light (Ronzani Cerquiera and Chatain, 1991). Larvae remain swimming in the light period and therefore expend energy unnecessarily during the inactive feeding period.

Temperature affects most aspects of early larval fish development, and embryos and larvae tend to be more stenothermal than juveniles and adults (Blaxter, 1988; Rombough, 1988; Rombough, 1996; van Der Kraak and Pankhurst, 1996; Rombough, 1996). In terms of swimbladder inflation and survival, pre-metamorphosis snapper larvae have a narrow

window of tolerance to temperature (18-24°C), which resembles the known temperature range for spawning (Pankhurst et al., 1991; Battaglene, 1995; Chapter 3). Little is known of the effects of temperature on swimbladder inflation for other species (Battaglene, 1995). Swimbladder inflation of striped bass, *Morone saxatilis* was better at temperatures lower than those considered optimal for growth (Hadley et al., 1987). This may be because pre-swimbladder inflation larvae are larger at lower temperatures and therefore are stronger swimmers and more able to reach the water surface to gulp air (Gibson and Johnston, 1995). The optimal temperature range for successful swimbladder inflation and subsequent survival of the coldwater striped trumpeter, *Latris lineata* in hatchery tanks has recently been determined and is extremely narrow at 16-17°C (Trotter et al., in press). This latter result highlights further the importance of doing rigorous laboratory experiments for every fish species to determine optimal rearing conditions in intensive hatcheries.

Within the tolerable temperature range, larval growth and development increases as temperature is increased (Chapter 3). On the other hand, post-metamorphosis snapper can tolerate a wide temperature range from 4°C to 31.5°C (Lagler et al., 1962; Battaglene and Talbot, 1992; Fielder, unpublished data). Similar results have been demonstrated for other early stage sparids (Person Le-Ruyet and Verillaud, 1980; Foscarini, 1988; Tandler et al., 1989; Tandler, 1993; Mihelakakis and Kitajima, 1994; Mihelakakis and Yoshimatsu, 1998).

Performance of snapper larvae described in this thesis supports the view that in general, temperature has a much greater effect on larval fish performance than salinity (Rombough, 1996). Snapper larvae were remarkably euryhaline and successfully completed most of the critical developmental stages over a wide range of salinities (10-45‰). Swimbladder inflation, growth and survival were only compromised at the lower and upper extremes of the tested salinity range (Chapter 3). Low salinity reduces the buoyancy of larvae which in

turn can prevent larvae from swimming to the water surface to gulp air and inflate their swimbladders (Hadley et al., 1987; Battaglene and Talbot, 1990; Battaglene, 1995; Howell et al., 1998). In contrast, hypersaline environments can increase larval stress and consequently reduce the success of swimbladder inflation (Johnson and Katavic, 1984; Tandler et al., 1995).

Reduced growth and survival of fish larvae in hypo- and hyperosmotic environments is most likely due to increased metabolic demand to maintain homeostasis and/or the inability of fish to alter osmoregulatory mechanisms to adapt to the environmental osmolality (Hwang and Hirano, 1985; Alderdice, 1988; Chapter 6). The osmoregulatory capacity of snapper larvae is unknown and remains to be determined.

Calculi in the urinary bladder and kidneys have been observed in a number of intensively reared sparids including snapper, gilthead sea bream, red sea bream and sharpsnout seabream (Ueda et al., 1970; Modica et al., 1993; Battaglene, 1995; Favaloro and Mazzola, 2000; Chapters 3 and 4). In some cases the presence of urinary calculi was associated with swimbladder dysfunction and larval mortality (Ueda et al., 1970; Modica et al., 1993) but this association is rare. The formation of urinary calculi does however suggest that larvae are being reared in sub-optimal environmental conditions and/or larvae are experiencing a degree of physiological dysfunction. This is supported by the fact that incidence of urinary calculi in snapper increases as salinity and temperature are increased within the tolerable ranges but high temperature had a greater effect on calculi formation than salinity. Importantly, urinary calculi formed quickly and in high numbers of larvae after larvae were exposed to elevated temperatures which were ultimately lethal ($\geq 27^{\circ}C$) (Chapter 3). It is possible that snapper larvae died as a consequence of urinary calculi at high temperatures but it is more likely that metabolic processes of larvae were compromised as a result of supra-optimal temperatures; formation of calculi was a result of

impaired metabolism. As larvae increased in size and development around metamorphosis, calculi were not observed and may have been expelled or dissolved as fish metabolism and osmoregulation mechanisms developed.

The results described in this thesis (Chapters 2 and 3) demonstrate that the environmental regime needed to optimise production of healthy, well-developed fish in intensive hatcheries is a compromise between conditions which promote successful completion of developmental milestones and those which promote growth and survival.

Despite the fact that many species of marine fish larvae are reared intensively in hatcheries, and the widespread understanding of the benefits of providing optimal key physical conditions, the optimal conditions for larval rearing have been determined only for a very small number of species (Shepherd and Bromage, 1988). For the few species which have had their optimal physical parameters identified, initially in small-scale tanks and then validated in commercial-scale trials, such as European sea bass and gilthead sea bream, hatchery production is well established and major aquaculture industries have developed (Tandler, 1993; Shields, 2001). Interestingly, although the optima for photoperiod, salinity and temperature are species-specific (Blaxter, 1969, Alderdice, 1988, Howell et al., 1998) there are close similarities in optimal rearing regimes between the sparids, snapper (this thesis) and gilthead sea bream (Tandler, 1993; Chatain, 1997; Shields, 2001). This information may be useful when preliminary attempts are made to rear new species of temperate sparids but ultimately optimal conditions should be experimentally determined for each species.

Validation of results from small tank experiments in large, commercial-scale tanks is important. The dynamics of non-target, abiotic variables such as light intensity, turbulence and aeration, which affect larval fish performance (Chatain and Ounais-Guschemann, 1991; Opstad and Bergh, 1993; Huse, 1994; Battaglene, 1995; Cobcroft et al., 2001) can

differ according to tank size. Therefore the effects of photoperiod, salinity and temperature on larval performance may be more or less influenced by extraneous variables. Moreover, the ability to manage the environmental parameters (e.g. temperature) within necessary ranges on a large scale is influenced by tank size. "Real" production also generates data for extrapolation and estimation of potential number and costs of fingerlings produced from hatcheries. For example, labour and electricity can represent approximately 50% of the operating costs of marine fish hatcheries (Candreva et al., 1996). However, estimates of these costs and benefits of production from small-scale trials may not be appropriate for commercial culture due to the rigorous and often expensive (e.g. high labour input, inefficient heating) nature of small-scale tank experiments.

An understanding of the tolerance and performance of fish larvae to parameters such as salinity and temperature is not only essential to improve current hatchery practices but is also important when new, alternative cultural methods are being developed. Interest is increasing to develop extensive larval rearing methods for snapper larvae in outdoor ponds. Ponds are generally exposed to ambient environmental conditions and abiotic parameters can change diurnally and seasonally. Knowledge of the narrow window of temperature tolerance for early stage snapper larvae for example, will improve production by enabling strategic times of larval stocking into ponds.

8.3. Suitability of saline groundwater

A range of crustaceans, salt-tolerant freshwater fish and diadromous and euryhaline fish have been cultured experimentally or commercially in inland saline groundwater in the United States, Middle East and Australia (Forsberg et al., 1996; Ingram et al., 1996; Samocha et al., 1998; Allan and Fielder, 1999; Allan and Fielder, 2002; Ingram et al., 2002). Unlike seawater, which is relatively stable in ionic composition and ratios of the

major ions, the salinity, chemical composition and ratios of the major ions in saline groundwater can vary significantly from site to site and can differ from that of similar salinity seawater (Forsberg et al., 1996; Nulsen, 1999; Chapter 5). In particular, Ca^{2+}, Mg^{2+} and K^+ can be present in very low or high concentrations compared with seawater of similar salinity (Forsberg et al., 1996; Chapter 5).

Growth and survival of fish has varied with water source and species and most likely reflects the ability of the fish to osmoregulate effectively and obtain essential ions from the water or food (Sakamoto and Yone, 1978; Sanders and Kirschner, 1983; Lall, 1989; Gardaire et al, 1991; Wilson and El Naggar, 1992; Chapters 5 and 7). Potassium is present in very low concentrations in some Australian saline groundwater but diadromous species such as Atlantic salmon, Australian bass and barramundi have survived well (Allan and Fielder, 1999; Ingram et al., 2002; Chapter 5). These species are capable of surviving in seawater, and in freshwater with extremely low and variable ionic composition and clearly can adapt osmoregulatory or behavioural mechanisms to sequester essential ions under these different environmental conditions. In contrast, marine and euryhaline fish have not performed well in some saline groundwaters taken directly from the source (Forsberg et al., 1996; Chapter 5). Snapper died due to insufficient $[K^+]$ in saline groundwater from New South Wales. However, fortification of different saline groundwater with salts such as $CaCl_2$, NaCl and KCl has improved growth and/or survival of red drum (Thomas and Wolters, 1992; Stahl et al., 1995; Forsberg et al., 1996) and snapper (Chapter 5). Provided the K^+/Cl^- ratio in inland saline groundwater from New South Wales is greater than 0.007, survival and growth of snapper in tanks is similar to that in equivalent salinity seawater.

Supplementation of dietary NaCl has resulted in little or no improvement in growth and feed conversion of some diadromous species such as Atlantic salmon grown in freshwater and seawater (Shaw et al., 1975), and rainbow trout, *Oncorhynchus mykiss* grown in

freshwater (MacLeod, 1978). In contrast, weight gain and food conversion of the euryhaline red drum grown in freshwater was improved when the diet was supplemented with NaCl and KCl (Gatlin et al., 1992). In addition, increased dietary NaCl, NaCl-K or NaCl-Mg mixtures promote gill Na^+,K^+,ATPase activity and consequently improves the hypoosmoregulatory ability of several species of salmonid smolt prior to their transfer from freshwater to saltwater (Zuagg et al., 1983; Salmon and Eddy, 1990; Staurnes and Finstad, 2000). Supplementation of feed with KCl may also improve performance of snapper in K^+-deficient saline groundwater but this remains to be investigated.

The ability for fish to alter physiological or morphological mechanisms in response to low environmental ion concentrations, especially in saline water has received little research attention. Lamellar gill chloride cells proliferated in freshwater-adapted fish after exposure to artificially softened freshwater (Greco et al., 1995) or freshwater which was deficient in Ca^{2+} or Mg^{2+} (Laurent and Perry, 1991; Bijvelds et al., 1997; Perry, 1998). Also Ca^{2+} uptake increased and was correlated with proliferation of gill chloride cells in trout, *Salmo gairdneri* held in low Ca^{2+} freshwater (Perry and Wood, 1985). Very little information exists on the ability of marine teleosts to adapt osmoregulatory mechanisms in ion-poor saline water. Several dilution schedules of $[K^+]$ in saline groundwater from complete K^+ fortification to raw saline groundwater $[K^+]$ were evaluated but snapper did not acclimatise to low $[K^+]$ and died when the K^+/Cl^- ratio was less than 0.007 (Chapters 5 and 7). The effects of K^+ deficient saline groundwater on osmoregulation were determined during this study (Chapter 7) and it appears unlikely that snapper will be able to acclimate to saline groundwater with K^+/Cl^- less than 0.007.

8.4. Osmoregulation and chloride cell morphology in seawater

The ability for fish to osmoregulate in a wide range of salinities is an excellent attribute for their aquaculture in ponds as salinity can vary widely according to ambient conditions (Wu and Woo, 1983). Knowledge of the ability for a fish species to osmoregulate is therefore important when selecting suitable sites for their aquaculture.

Juvenile snapper, like several other species of sparids including red sea bream, silver sea bream, *Sparus sarba*, black sea bream, *Mylio macrocephalus* and gilthead sea bream, is euryhaline and can osmoregulate in low salinity (near-isoosmotic) and hyperosmotic environments (Woo and Fung, 1981; Mancera et al., 1993; Kelly and Woo, 1999; Kelly et al., 1999; Chapter 6). After abrupt transfer from seawater to higher or lower salinity environments initial perturbations in osmolality and blood electrolyte (Cl^-, Na^+, K^+) concentration occurs however homeostasis is restored quickly, usually within 24-72 h (Woo and Fung, 1981, Mancera et al., 1993; Kelly and Woo, 1999; Kelly et al., 1999; Chapter 6). Fish restore homeostasis by altering drinking rate (increases as environmental osmolality is increased and vice versa), stress hormone levels and functions of the osmoregulatory surfaces, especially those of the branchial epithelia (Tytler and Blaxter, 1988; Ura et al., 1996; Miyazaki et al., 1998; Wendelaar Bonga, 1997; Brown et al., 2001; Hwang and Hirano, 1985; Perry, 1998; Kelly and Woo, 1999).

Snapper gill epithelium contains two distinct types of gill chloride cells, filament and lamellar chloride cells which also occur in several species of diadromous and euryhaline fish (Uchida et al., 1996; Hiroi et al., 1998; Sasai et al., 1998; Hirai et al., 1999; Uchida et al., 2000; Chapter 6). In contrast only filament chloride cells have been observed in black sea bream and silver sea bream, which can osmoregulate in very low environmental salinities (~6‰) (Kelly and Woo, 1999; Kelly et al., 1999). This demonstrates that

morphological and physiological mechanisms used for adaptation to changes in salinity are species-specific and can differ within closely related species.

Gill filament chloride cells in snapper displayed typical morphological changes in response to decreased and increased environmental salinity. In near-isoosmotic salinity (15‰) filament chloride cells decreased in number and size but in a hypersaline (45‰) environment filament cells increased in size (Yoshikawa et al., 1993; Uchida and Kaneko, 1996; Uchida et al., 1997; Wales, 1997; Sasai et al., 1998; Hirai et al., 1999; Uchida et al., 2000).

In contrast to the current views on the functions of gill lamellar chloride cells and their role in adaptation of fish to a freshwater environment (Laurent and Dunel, 1980; Perry and Laurent, 1989; Perry, 1997; Perry, 1998), gill lamellar chloride cells may also play a role in salt excretion in snapper (Chapter 6). Alternatively, snapper may retain a degree of hypoosmotic adaptability (Sasai et al., 1998). In addition, the degree of swimming activity of fish can affect the presence of gill lamellar chloride cells in marine fish (Hughes and Umezawa, 1983; Sala et al., 1987). The activity of tank-reared and wild snapper may vary with consequent differences in chloride cell morphology but this remains to be investigated. The functions of snapper gill lamellar chloride cells after exposure to a range of salinities may be understood further through electron-microscopic observations of cell ultrastructure and should be investigated.

Investigation of snapper osmoregulation in a range of salinities was done at a single target temperature (~21°C). Because temperature and salinity can have significant interactive effects on fish (Kinne, 1963; see Chapter 1) and temperature varies widely in aquaculture ponds, further research should be done to investigate the interactions of a range of salinities and temperatures on osmoregulation of snapper.

8.5. Osmoregulation and chloride cell morphology in saline groundwater

Maintenance of osmotic homeostasis in saltwater-adapted teleosts depends on the active secretion of excess intra- and extracellular NaCl (Alderdice, 1988; McCormick, 1995). This occurs through branchial chloride cells in metamorphosed fish and is driven by two active processes involving Na^+,K^+-ATPase and a $Na^+/K^+/2Cl^-$ cotransporter. Both Na^+,K^+,ATPase and the $Na^+/K^+/2Cl^-$ cotransporter are dependent on K^+ and, removal of K^+ from the basolateral side of chloride cells can rapidly inhibit the active transport of Cl^- (reviewed by Marshall and Bryson, 1998). Although some K^+ can be sequestered from the environment through branchial diffusion, the majority of K^+ is obtained from ingested seawater and food (Sakamoto and Yone, 1978; Sanders and Kirschner, 1983; Wilson and El Naggar, 1992). When [K^+] was removed completely or partially from environmental seawater the active processes of Na^+ and Cl^- secretion were inhibited in excised gill epithelia (Sanders and Kirschner, 1983; Marshal and Bryson, 1998). A similar response was observed in snapper exposed to K^+ deficient saline groundwater with a K^+/Cl^- ratio of 0.001 (Chapter 7). Snapper serum osmolality, [Cl^-] and [Na^+] increased with a concurrent decrease in serum [K^+] and snapper died. Provided the environmental K^+/Cl^- ratio in saline groundwater from New South Wales is $\geq$ 0.007 osmoregulation of snapper was similar to that in equivalent salinity seawater.

Proliferation of lamellar chloride cells is known to occur in freshwater fish exposed to low environmental Ca^{2+} and Mg^{2+} (Laurent and Perry, 1991; Greco et al., 1995; Bijvelds et al., 1997; Perry, 1998). However gill filament and lamellar chloride cells of snapper did not change in size or number following exposure to K^+ deficient saline water (Chapter 7). This result provides further evidence that both types of chloride cells are responsible for salt excretion in snapper and that the principal route of K^+ absorption in saltwater-adapted snapper is via the gut from ingested seawater.

8.6. Implications for snapper production

The research reported in this thesis describes the optimal photoperiod, salinity and temperature regime for intensive larval rearing of snapper in small-scale tank experiments (Chapters 2 and 3). The optimal "new" regime is different to the original "best-practice" regime used in most Australian snapper hatcheries. Commercial-scale evaluation/validation of the "new" larval rearing regime confirmed that snapper growth was approximately 1.5-times greater than in the original "best-practice" regime (Chapter 4). The implications for snapper fingerling production are that more fish can be produced annually and cost of production can be reduced. Labour cost is relatively fixed in intensive hatcheries regardless of the number of fingerlings produced, therefore the cost of labour to produce each fish will be reduced as the number of fish produced increases. Labour costs associated with artemia production will also be reduced because bigger, faster-growing larvae can be weaned earlier from live feeds onto a pellet diet. Weaned juvenile fish will then be transferred from the hatchery to nursery systems thus allowing another hatchery cycle to start. Snapper eggs are available year-round therefore approximately eleven hatchery cycles are possible with the "new" regime compared with seven hatchery cycles with the original "best-practice" regime.

Further improvements in larval rearing of snapper may be achieved if new larval feeding schedules can be developed to reduce the reliance on artemia feeding in intensive systems and alternative extensive larval rearing methods can be developed. As demonstrated for another euryhaline species, mulloway, *Argyrosomus japonicus* reared at the Port Stephens Fisheries Centre, the optimal larval rearing strategy may involve an initial rearing phase in the controlled, intensive hatchery followed by a secondary phase to metamorphosis in ponds (Fielder et al., 1999). Replacement of artemia with copepods

cultured in ponds and early weaning of snapper larvae with formulated diets in intensive hatchery systems, as well as evaluation of extensive larval rearing methods for snapper are the focus of a current large research project undertaken by NSW Fisheries with support from the Fisheries Research and Development Corporation and The Aquafin Cooperative Research Centre. This research program was developed following results presented in this thesis.

The research described in this thesis has provided an experimental base for new culture conditions to greatly improve survival and growth of snapper in inland saline groundwater. These new culture conditions will improve the aquaculture potential of snapper in Australia. In response to results of the present investigation , NSW Fisheries and Murray Irrigation Limited have constructed and opened a new research facility, The Inland Saline Aquaculture Research Centre at Wakool in western New South Wales. The objective of this centre is to conduct research and development to identify business opportunities for aquaculture in inland saline groundwater evaporation basins. Performance of four species including snapper, mulloway, silver perch, *Bidyanis bidyanis* and black tiger prawn, *Penaeus monodon* will be evaluated in purpose-built ponds and optimal management strategies developed for viable, sustainable aquaculture. If successful, the centre will also provide a vital link in technology transfer between research and new industry stakeholders by operating as a model, demonstration farm.

8.7. General Summary

1. **The optimal environmental regime for intensive rearing of snapper larvae is a compromise between conditions which promote successful completion of developmental milestones and those which promote growth and survival.**

- Swimbladder inflation of larvae was greatest at a photoperiod of 12 h light: 12 h dark; once swimbladders were inflated development and growth was best at 18 h light: 6 h dark.

- Snapper larvae were remarkably euryhaline and survived from 10‰ to 45‰ but swimbladder inflation, development, growth and survival was best from 20‰ to 35‰.

- pre-metamorphosis snapper larvae have a narrow window of tolerance to temperature (18-24°C) in terms of survival and and swimbladder inflation; development and growth was best at 24 °C

2. **Commercial-scale production confirmed results of small-scale experiments to identify the optimal photoperiod, salinity and temperature regime for intensive snapper larval rearing. Hatchery production of fingerlings will be significantly improved.**

    - Growth of snapper larvae in the "new" regime of photoperiod (12L:12D to swimbladder inflation, then 18L:06D), salinity (20-35‰) and temperature (24°C)

was 1.5 times greater than that in the previous "best-practice" regime of photoperiod (14L:10D), salinity (35‰) and temperature (21°C).

- Approximately 11 hatchery batches/year are possible with the "new" regime compared with 7 hatchery batches/year for the previous "best-practice" regime.

- Hatchery costs of production will be reduced as the number of fingerlings produced is increased

3. **Saline groundwater from inland New South Wales is suitable for growth and survival of juvenile snapper in tanks provided [K^+] is increased.**

- Composition of the major ions in raw saline groundwater is similar to coastal seawater of the same salinity with the exception that saline groundwater contains only 4.5% of the potassium concentration in coastal seawater.

- Snapper died in 2-4 d after transfer from coastal seawater to raw saline groundwater.

- Fortification of raw saline groundwater with KCl improved water quality and provided the [K^+] was 40% of that found in same salinity coastal seawater (K^+/Cl^- ratio = 0.007) snapper survived and grew. When [K^+] was 60-100% of that found in same salinity coastal seawater (K^+/Cl^- ratio = 0.01-0.018) growth of snapper in tanks was similar to coastal seawater.

- Snapper did not acclimate to raw saline groundwater after extended periods of gradual K^+ dilution.

4. **Juvenile snapper are euryhaline and can osmoregulate in near-isoosmotic and hyperosmotic environments.**

- Serum osmolality, $[Na^+]$,$[K^+]$ and $[Cl^-]$ increased and decreased (except $[K^+]$) after 24 h following transfer from 30‰ to 45‰ and 15‰, respectively.

- The serum chemistry changes were transient and had returned to near initial levels after 168 h in 45‰ and 15‰.

- Restoration of homeostasis was concomitant with changes in morphology of chloride cells

- Gill filament and lamellar chloride cells were observed in juvenile snapper but filament chloride cells were more abundant than lamellar chloride cells.

- In 45‰ filament chloride cells increased in size by 1.4-times in after 168 h but the number of filament and chloride cells did not change

- In 15‰ the number of filament chloride cells and the size of both filament and lamellar chloride cells had decreased after 72 h.

- Both filament and lamellar chloride cells are responsible for excretion of excess salt from snapper in hyperosmotic environments.

5. **Juvenile snapper can osmoregulate in saline groundwater when the [K^+] is 40% or greater (K^+/Cl^- ratio is $\geq$ 0.007) of that found in similar salinity coastal seawater (30-35‰).**

- Serum osmolality, [Cl^-], [Na^+] and [K^+] in saline groundwater fortified with 40% and 100% K^+ were generally similar to the initial levels during 168 h after transfer from coastal seawater.

- Serum osmolality, [Cl^-] and [Na^+] of fish transferred from coastal seawater to raw saline groundwater increased rapidly, whereas serum [K^+] decreased rapidly after 72 h and fish became moribund.

- Morphology of chloride cells did not change in response to low environmetal [K^+].

8.8. References

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Survival and growth of Australian snapper, *Pagrus auratus*, in saline groundwater from inland New South Wales, Australia

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Abstract

Australia has extensive resources of inland saline groundwater, which may be suitable for culture of marine fish. This study assessed the suitability of saline groundwater, which was pumped from a shallow aquifer into an evaporation pond near Wakool in western New South Wales, for growth and survival of juvenile snapper, *Pagrus auratus*. Five experiments were conducted. The first showed that snapper (31 g) did not commence feeding, lost equilibrium of buoyancy and became moribund within 3 days after transfer from coastal seawater (diluted to 19‰ with rainwater) to saline groundwater (19‰). Potassium concentration of diluted coastal seawater and groundwater (both 19.6‰) was 203 and 9.2 mg l^{-1}, respectively, while the concentration of most other major ions was similar in water from both sources. In the second experiment, groundwater of 21‰ salinity was fortified with potassium (as KCl) to provide 25%, 50% or 100% of the concentration of potassium found in coastal seawater of 21‰ salinity. Survival and feeding and swimming behaviour of snapper (1.5 g) held in tanks for 8 days were the same in 50% and 100% potassium-fortified treatments as in coastal seawater controls. However, snapper held in groundwater fortified with only 25% potassium, or raw saline groundwater became moribund after 4 and 2 days, respectively. During the third 42-day experiment, growth, survival and food conversion of juvenile snapper (4.0 g) were the same in diluted coastal seawater

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(20‰) and groundwater (20‰) provided the level of potassium in the groundwater was increased to within 60–100% of the concentration in coastal seawater. During the fourth experiment, juvenile snapper were acclimatised to raw saline groundwater by transferring fish from fortified groundwater with initial potassium levels of 100% of that in coastal seawater, to groundwater with 10% lower potassium levels every 3.5 days or 20% lower levels every 7 days. A further treatment where snapper were transferred from groundwater fortified initially with potassium levels of 60% of coastal seawater, to groundwater with 20% lower potassium levels every 3.5 days was included. When potassium was reduced to 20% of the concentration in coastal seawater, in all treatments, fish became moribund. Results from the fifth experiment, where groundwater was fortified with either KCl or NaCl at equivalent chloride levels, confirmed that potassium and not chloride ions were responsible for improvement in groundwater. Our results demonstrate that saline groundwater from Wakool, fortified with KCl is a suitable medium for growing snapper juveniles in tanks.

Keywords: Snapper; *Pagrus auratus*; Aquaculture; Saline groundwater

1. Introduction

Snapper, *Pagrus auratus*, occurs in temperate waters in all Australian states, Lord Howe Island and Norfolk Island (Battaglene and Bell, 1991) and is the target of recreational and commercial fisheries (Bell et al., 1991; Francis, 1994). Juveniles usually live in estuaries, while adults inhabit coastal and offshore waters. Catches, particularly from the Australian east coast, are declining (ABARE, 1998) and, as a consequence, programs to develop aquaculture protocols for snapper are current in temperate Australia (Battaglene and Fielder, 1997).

Australian snapper is the same species as the Japanese red sea bream (*P. auratus* = *P. major*; Paulin, 1990), which has been cultured successfully in Japan for 30 years, using intensive larval rearing followed by growout in sea-cages (Foscarini, 1988; Battaglene and Bell, 1991). It is seen as an excellent candidate species for aquaculture and possibly stock enhancement in Australia.

Commercial snapper hatcheries and floating sea-cage farms are now operating in New South Wales (NSW), South Australia and Queensland using techniques described by Battaglene (1995), Battaglene and Talbot (1992) and Quartararo (1996) or adapted from those used in Japan to rear red sea bream. However, expansion of a sea-cage-based snapper farming industry in Australia may be limited by the lack of sites with suitable water quality, water depth and proximity to land-based infrastructure (Ogburn, 1996). Approval of sea-cage farms by relevant government bodies is also affected by conflict with other waterway users and perceived concerns about environmental impact.

Alternative, additional sites may be available for farming marine species if the large reserves of saline groundwater occurring in inland areas of Australia can be utilised. Shallow saline water tables are migrating towards the soil surface in many semiarid areas especially where crops are irrigated (Blackwell, 1999). Crop production is affected adversely and natural vegetation is destroyed. In some inland areas, upward migration of the water table is addressed by pumping saline groundwater into a series of large evaporation ponds, which range in size from 2 to 30 ha (Ingram et al., 1996; Allan and

Fielder, 1999). The salt concentration of this water increases progressively as it flows from one pond to the next and eventually crystalline salt is deposited.

The largest saline groundwater evaporation scheme in Australia is the Wakool–Tullakool Subsurface Drainage Scheme at Wakool, NSW, where approximately 13,000 Ml $year^{-1}$ of saline groundwater is pumped into 1600 ha of evaporation ponds (Ruello, 1996). The major ions found in seawater, in order of magnitude, are chloride (Cl^-), sodium (Na^+), sulphate (SO_4^{2-}), magnesium (Mg^{2+}), calcium (Ca^{2+}) and potassium (K^+) (Spotte, 1979). In saline groundwater from Wakool, the concentration of Cl^-, Na^+, SO_4^{2-} and Mg^{2+} is similar to seawater of the same salinity; however, the concentration of Ca^{2+} and K^+ in Wakool saline groundwater is much higher and lower, respectively, than seawater of the same salinity.

Saline groundwater has been used successfully in the United States and the Middle East to culture a range of algae, crustaceans and finfish, such as tilapia, red drum, sea bream, eels and channel catfish (Forsberg et al., 1996; Ingram et al., 1996; Samocha et al., 1998). In Australia, saline groundwater from shallow and deep aquifers has been suitable for growth and survival of euryhaline finfish, such as silver perch, *Bidyanus bidyanus*, Australian bass, *Macquaria novemaculeata*, barramundi, *Lates calcarifer* and Atlantic salmon, *Salmo salar* (Allan and Fielder, 1999; Gooley et al., 1999). To date, attempts have not been made to culture relatively stenohaline marine finfish like snapper in Australian saline groundwater.

The aim of this study was to assess the suitability of saline groundwater collected from an evaporation pond at Wakool, for growth and survival of juvenile snapper.

2. Materials and methods

Five laboratory bioassays were conducted at the NSW Fisheries Port Stephens Fisheries Centre (PSFC) from July 1997 to April 1998.

2.1. Source of juvenile snapper

Fertilised snapper eggs were obtained from first generation hatchery-reared (G1) snapper held in tanks at PSFC. Snapper either spawned spontaneously or were induced to spawn using hormones and techniques described by Lee et al. (1986).

The first group of juvenile snapper was produced after larvae were cultured for 57 days in a 2000-l tank using techniques similar to those described by Battaglene and Talbot (1992). After this time, approximately 1000 juvenile snapper were harvested from the tank and 200 were placed into each of five 500-l floating cages placed within a 100,000-l outdoor, in-ground concrete tank. While in the cages, juvenile snapper were fed a 45% protein, 1–4 mm crumble diet (Kinta, Australia). Ambient estuarine water (range, 23–33‰, 14–25°C) was pumped into the tank for 4 h each day to replace approximately 20% of the total tank volume.

The second group of juvenile snapper was produced after larvae were cultured for 32 days in three 10,000-l outdoor, concrete tanks using "greenwater" culture techniques. After this time, juvenile snapper were harvested and ongrown in 10,000-l fibreglass

tanks until used in experiments. Estuarine seawater (30–35‰) was supplied constantly with a flow-through rate of approximately 200 l h^{-1}. While in the tanks, juvenile snapper were fed 400 and 800 μm, 52% protein "ML powered" weaning diet.

2.2. Source of saline groundwater and coastal seawater

Saline groundwater was collected on two occasions from an individual 30 ha evaporation pond, which was part of the Murray Irrigation Limited, Wakool–Tullakool Subsurface Drainage Scheme (35°28′S, 144°26′E) and transported in tanks by road to the PSFC. The saline groundwater collected first (collection 1) had a salinity of 19‰ and was stored until needed for experiments in 10 × 30-l drums. The salinity increased to 21‰ following evaporation during this study. The saline groundwater collected second (collection 2) had a salinity of 30‰ and was stored in a 10,000-l storage tank, which was covered by a lid to exclude light and reduce evaporation.

Coastal seawater was collected from an ocean beach at Port Stephens (32°45′S, 152°04′E), transported by road to the PSFC and stored in a 10,000-l tank.

Generally, the chemistry of the saline groundwater (SG) from Wakool and coastal seawater (CS) of the same salinity was similar (Table 1). Both SG and CS were dominated by chloride ions, which constituted approximately 56% of the total salinity. Of the other major elements, in order of magnitude, the concentrations of sodium and sulphate in SG were 80% of that in CS, whereas the concentrations of magnesium and calcium were 1.5 and 2.5 times greater, respectively in SG than in CS. There was a major difference in the concentration of potassium, which was present in SG at only 4.5% of the concentration in the same salinity CS. There were mostly small differences in concentration of minor elements, such as heavy metals, in SG and CS (Table 1).

2.3. Experiment 1: effect of raw saline groundwater

This experiment was designed to determine the effect of raw saline groundwater on short-term survival and behaviour of juvenile snapper. Two water treatments were tested: raw saline groundwater (SG) and coastal seawater (CS).

The experiment was conducted in 6 × 60-l glass aquaria (three replicates per treatment). Each aquarium had a simple airlift-driven biofilter, which occupied approximately 20% of the volume. Tanks were covered with black plastic to reduce light intensity, but a window covered by a black plastic flap, allowed observation of experimental fish.

Three randomly positioned tanks were filled with each experimental treatment solution (Table 2). Each tank was then stocked with four randomly selected juvenile snapper (mean wet weight 31.3 g, $n = 10$; group 1), which had been acclimatised from 30‰ to 19‰, 24 h earlier. No water was exchanged during the experiment, which was terminated after 4 days.

2.4. Experiment 2: short-term effect of fortified saline groundwater

This experiment was designed to determine the effect of fortifying raw saline groundwater with K^+ (as KCl) on short-term survival and behaviour of juvenile

Table 1
Water chemistry of coastal seawater and saline groundwater from the Wakool-Tullakool Subsurface Drainage Scheme, New South Wales, Australia

Element or chemical	Coastal seawater		Coastal seawater diluted[a]		Raw groundwater	
Salinity (‰)	35.3		19.6		19.6	
pH	8.1		–		7.9	
Alkalinity, ($mg\ l^{-1}$) as $CaCO_3$	114		63		195	
Major ions	($mg\ l^{-1}$)	ion/Cl^- ratio	($mg\ l^{-1}$)	ion/Cl^- ratio	($mg\ l^{-1}$)	ion/Cl^- ratio
Chloride	20,000	1.000	11,105	1.000	11,000	1.000
Sodium	9470	0.474	5258	0.474	4210	0.383
Sulphate	2500	0.125	1388	0.125	1100	0.100
Magnesium	1000	0.050	555	0.050	820	0.075
Potassium	365	0.018	203	0.018	9.2	0.001
Calcium	364	0.018	202	0.018	504	0.046
Minor elements						
Fluoride	0.76		0.42		0.44	
Total PO_4, ($mg\ l^{-1}$) as P	< 0.10		–		< 0.10	
Aluminium	< 0.001		–		0.007	
Boron	4.4		2.4		0.38	
Bromide	72		40		34	
Copper	0.012		0.007		0.007	
Iodide	< 0.05		–		0.16	
Iron	< 0.05		–		< 0.05	
Lead	< 0.001		–		< 0.001	
Lithium	0.17		0.09		0.07	
Manganese	< 0.001		–		0.001	
Mercury	< 0.001		–		< 0.001	
Molybdenum	0.012		0.007		0.002	
Strontium	7.6		4.2		9.1	
Zinc	0.004		0.002		< 0.001	

[a]Values calculated from coastal seawater at 35.3‰.

snapper. For this and subsequent experiments, stock solutions for experimental treatments, which included K^+ fortification, were prepared by dissolving analytical grade KCl to give the required K^+ concentration (Table 2). Coastal seawater was diluted using rainwater. The following water treatments (all 21‰) were tested:

(a) raw saline groundwater (SG raw),
(b) 100% K^+-fortified saline groundwater (SG100),
(c) 50% K^+-fortified saline groundwater (SG50),
(d) 25% K^+-fortified saline groundwater (SG25),
(e) coastal seawater control (CS).

Table 2
Experimental water treatments, target ion concentrations and quantities of added salts (KCl; NaCl). Coastal seawater (CS) and saline groundwater (SG) were collected from Port Stephens and Wakool, New South Wales, Australia, respectively

	Treatment	K^+ concentration (mg l^{-1})	Amount of KCl added (mg l^{-1})		
Experiment 1 (19.0‰)[a]	CS[b]	197	0		
	SG raw	9	0		
Experiment 2 (21.0‰)[a]	CS[b]	217	0		
	SG100	217	395		
	SG50	109	188		
	SG25	54	84		
	SG raw	10	0		
Experiment 3 (20.0‰)[c]	CS[b]	207	0		
	SG100	207	378		
	SG80	166	300		
	SG60	124	220		
	SG40	83	141		
	SG100-30[d]	310	565		
Experiment 4 (21.5‰)[c]	SG100	223	407		
	SG90	200	363		
	SG80	178	321		
	SG70	156	279		
	SG60	134	237		
	SG50	112	195		
	SG40	90	153		
	SG30	67	109		
	SG20	45	67		
	SG10	22	23		
	SG raw	10	0		
		K^+ concentration (mg l^{-1})	Added Cl^- concentration (mg l^{-1})	Amount of KCl added (mg l^{-1})	Amount of NaCl added (mg l^{-1})
Experiment 5 (21.0‰)[c]	SGK100	207	188	395	0
	SGCL100	10	188	0	310

[a]Undiluted saline groundwater; collection 1.
[b]Coastal seawater diluted with rainwater from 35.3‰.
[c]Saline groundwater diluted with rainwater from 30‰; collection 2.
[d]Undiluted saline groundwater; collection 2.

The experiment was conducted in 15 × 2-l opaque, plastic buckets with lids (three replicates per treatment). A total of 1.5 l of each experimental water treatment was placed into three randomly selected buckets. Approximately 85% of the bucket water volume was poured to waste each day and replaced with new treatment water. Air was supplied to each container through a 1-ml glass pipette at 50 ml min^{-1}. Fluorescent light

was provided at the surface of each container at 3.0 μmol s^{-1} m^{-2} on a 14:10 h light:dark photoperiod.

Each bucket was stocked with four randomly selected 61 dah juvenile snapper (mean wet weight 1.5 g, $n = 4$; group 2), which had been acclimatised from 30‰ to 21‰, 24 h earlier. The experiment was terminated after 8 days.

2.5. Experiment 3: long-term effect of fortified saline groundwater

This experiment was designed to determine the effect of fortifying raw saline groundwater with K^+ on long-term survival and growth of juvenile snapper. The water treatments (all 20‰, unless otherwise stated; Table 2) tested were:

(a) 100% K^+-fortified saline groundwater (SG100),
(b) 80% K^+-fortified saline groundwater (SG80),
(c) 60% K^+-fortified saline groundwater (SC60),
(d) 40% K^+-fortified saline groundwater (SG40),
(e) 100% K^+-fortified saline groundwater, 30‰ (SG100-30),
(f) coastal seawater control (CS).

The experiment was conducted in 24 × 100-l conical bottomed tanks with black sides and white bottoms (four replicates per treatment). Each tank was part of an independent, recirculating system operated with an internal 500-μm mesh-covered standpipe, an external airlift pump and biofilter (described by Fielder and Bardsley, 1999) and an internal, fully immersed, 2.75-l airlift-driven biofilter, which was filled with bioballs (Academy Plastics, Australia). Incandescent light was provided at the surface of each tank at 23 ± 1.3 μmols^{-1} m^{-2} (mean ± S.E.; $n = 30$ tanks) on a 14:10 h light:dark photoperiod. Approximately 5% of the experimental tank water was exchanged each day and salinity was maintained by adding rain water when needed.

Each experimental water treatment was placed into four randomly selected tanks. Juvenile snapper (97 dah; group 2) were anaesthetised with 20 mg l^{-1} of benzocaine in the stock tank, and a random sample of fish was weighed to provide an estimate of fish weight (3.9 ± 0.8 g, ± S.D., $n = 20$). Fish were then weighed individually and six fish of similar size were stocked into each experimental tank. Fish were fed to satiation daily by hand at 0900 and 1500 h with 52% protein "ML-powered" weaning pellets. The experiment was terminated after 42 days.

2.6. Experiment 4: acclimation of juveniles to raw saline groundwater

This experiment was designed to determine if juvenile snapper could be acclimatised from KCl-fortified saline groundwater to raw saline groundwater by rapid or slow dilution of the K^+ concentration. The acclimation treatments (all 21.5‰; Table 2) tested were:

(a) 100% K^+-fortified saline groundwater, no reduction in K^+ (SG100-C);
(b) 100% K^+-fortified saline groundwater, K^+ reduced by 10% every 3.5 days (SG100-slow);

(c) 100% K^+-fortified saline groundwater, K^+ reduced by 20% every 7 days (SG100-rapid);
(d) 60% K^+-fortified saline groundwater, K^+ reduced by 20% every 7 days (SG60-rapid).

The experimental buckets, daily water management and lighting for this experiment were the same as those described for Experiment 2. Each experimental water treatment was placed into three randomly selected buckets. Three randomly selected 109 dah juvenile snapper (mean wet weight 3.2 ± 0.6 g, $\pm$S.D., $n = 10$; group 2) were then placed into each bucket. The experiment was terminated after 33 days.

2.7. Experiment 5: effect of K^+ or Cl^-

This experiment was designed to determine whether the improvement in performance of juvenile snapper in raw saline groundwater fortified with KCl was due to an increase in K^+ or Cl^- concentration. The water treatments (21‰; Table 2) tested were:

(a) 100% K^+-fortified saline groundwater (SGK100),
(b) saline groundwater fortified with analytical grade NaCl to provide the same Cl^- concentration as occurred in treatment (a) (SGCL100).

The experiment was conducted in 6×4-l glass beakers (three replicates per treatment), each filled with 3.4 l. The daily water management and lighting were the same as those described for Experiment 2. Each experimental water treatment was placed into three randomly selected beakers. Each tank was then stocked with three randomly selected 168 dah juvenile snapper (mean wet weight 21.9 ± 4.5 g, $\pm$S.D., $n = 6$ tanks; group 2). The experiment was terminated after 4 days.

2.8. Measurement of survival, fish behaviour, growth and feeding

For Experiments 1, 2, 4 and 5 snapper were fed to satiation by hand with 54% protein "ML-powered" pellets once each day at approximately 0900 h when the number of surviving fish and the number of fish feeding were counted, and swimming behaviour was assessed. Moribund and dead fish were removed from the tanks as soon as they were noticed. In Experiments 2, 4, and 5, the feeding and swimming behaviour of individual fish was scored on a scale of 5 (normal) to 1 (abnormal).

In Experiment 3, all moribund and dead fish were removed from the tanks as soon as they were noticed. Stocking density was maintained in each tank by replacing dead fish with fin-clipped fish of similar weight. A group of replacement fish for this purpose were pelvic fin-clipped for identification 7 days before the start of the experiment, treated for 48 h with 50–100 mg l^{-1} of oxytetracycline hydrochloride to prevent infection of wounds and then held in a 400-l tank with flow-through estuarine seawater (30–32‰). Replacement fish were not used in estimates of weight gain. Feed consumption was recorded daily. Weight gain was recorded every 14 days. After 42 days, fish were harvested and the percentage survival, mean fish wet weight, mean adjusted

Table 3
Ranges of water quality parameters for Experiments 1–5

Experiment	DO_2 (mg l^{-1})	Salinity (‰)	pH	Temperature (°C)
1	5.6–6.4	18.0–19.0	8.3–8.5	20.5–21.6
2	4.0–6.0	20.8–21.5	7.4–8.7	20.3–24.2
3	6.2–7.9	19.7–20.9 (target 20), 29.7–31.1 (target 30)	7.4–8.1	22.1–24.4
4	4.0–7.5	21.5–23.3	7.7–8.1	21.9–22.7
5	5.1–6.9	21.1–21.7	7.7–8.1	21.9–22.6

biomass gain = [final total biomass + weight of dead fish] − [initial total biomass + weight of replacement fish], mean fish dry weight and food conversion ratio (FCR) were calculated from data recorded for each tank.

2.9. Daily water measurement

In all experiments salinity, temperature, pH and dissolved oxygen were measured daily using a water quality meter (Horiba U-10, Japan) (Table 3). In Experiment 3, total ammonia–nitrogen (< 1.0 mg l^{-1}) was measured daily in at least one replicate tank from each treatment with a rapid test kit (E. Merck, Model 1.08024, Germany). Light intensity (photosynthetically active radiation) was measured with a light meter (LI-COR, model Li-1776, USA).

2.10. Statistical analyses

Data were assessed for homogeneity of variance using Cochran's test (C; Winer, 1971). For Experiment 3, data for survival ($P = 0.0001$, $C = 0.8$) and FCR ($P = 0.003$, $C = 0.7$) were heterogeneous and could not be transformed to satisfy the assumption of homogeneity of variance. Experiments 2–5 were designed for analysis using single factor analysis of variance (ANOVA). Where significant differences were found, means were compared by the Student–Newman–Keuls test (SNK). Statistical analyses were conducted using Statgraphics Version 5.0 (STSC, USA).

3. Results

3.1. Experiment 1: effect of raw saline groundwater

Snapper held in SG were actively swimming for 1 day following transfer from CS, but did not feed vigorously. After 2 days, one fish had died and two fish had lost equilibrium of buoyancy and were floating upside down, while the remaining fish were lethargic and displayed no flight response when challenged with external stimuli. This trend continued, and after 4 days, all fish in the SG were removed from treatment tanks

following loss of equilibrium of buoyancy, or had died. Snapper held in CS treatments for 4 days all survived and were actively swimming and feeding during this time.

Effort was made in this and all subsequent experiments to remove moribund snapper from the treatment solutions as soon as they were noticed and return them to coastal seawater; however, some snapper died prior to removal from treatment solutions and no fish survived after transfer to seawater.

3.2. Experiment 2: short-term effect of fortified saline groundwater

After 8 days, all fish that were held in CS, SG50 and SG100 treatments had survived and there was no significant difference ($P > 0.05$) in swimming and feeding behaviour (Table 4). No fish survived for more than 2 and 4 days when held in the SG raw and SG25 treatments, respectively.

3.3. Experiment 3: long-term effect of fortified saline groundwater

Survival of snapper was high and not significantly different ($P > 0.05$) for all water treatments (Table 5). A total of three fish died during the experiment as a result of jumping from the tanks; one fish in one tank of the SG60 treatment, and two fish in one tank of the SG40 treatment.

Snapper grew in all water treatments; however, the final wet weight of snapper grown in the SG40 treatment was significantly lower ($P < 0.05$) than that of snapper grown in CS, SG60, SG80, SG100 or SG100-30, which did not differ (Table 5). Multiple comparisons of means for final dry weight failed to clearly separate treatment differences; however, the final dry weight of snapper grown in SG40 was significantly ($P < 0.05$) lower than that of snapper grown in SG60, SG80, SG100 or SG100-30, which were similar (Table 5).

The amount of feed consumed and FCR were affected significantly ($P < 0.05$) by the concentration of K^+ in the saline groundwater; however, multiple comparisons of the means failed to clearly separate the main treatment effects. The fish grown in the SG40 consumed up to 28.8% less feed than snapper grown in the SG100 treatment. Feed consumption was similar for the other treatments (Table 5). The FCR of snapper was

Table 4
Final survival, swimming and feeding behaviour[a] of juvenile snapper *P. auratus* held for 8 days in saline groundwater fortified with KCl to provide potassium at different concentrations as occurred in equivalent salinity coastal seawater (Experiment 2)

Treatment	Survival (%)	Swimming behaviour	Feeding behaviour
Coastal seawater	100±0	5.0±0	4.8±0.3
SG 100% K^+	100±0	4.8±0.3	4.7±0.3
SG 50% K^+	100±0	4.8±0.3	4.1±0.5
SG 25% K^+	0±0	0±0	0±0
SG raw	0±0	0±0	0±0

Data are means ± standard errors ($n = 3$ tanks).

[a] Feeding and swimming behaviour of individual fish was scored on a scale of 5 (normal) to 1 (abnormal).

Table 5
Growth performance, survival and food conversion of juvenile snapper *P. auratus* grown for 42 days in saline groundwater fortified with KCl to provide potassium at different concentrations as occurred in equivalent salinity coastal seawater (Experiment 3)[a]

Treatment		Salinity (‰)	Initial wet weight (g)	Final wet weight (g)	Final dry weight (g)	Adjusted wet weight gain[b] (g)	Survival (%)	Feed input[c] (g)	FCR[d]
Seawater	(CS)	20	4.1 ± 0.1^{x}	12.5 ± 0.6^{x}	3.8 ± 0.2^{y}	51.0 ± 3.4^{x}	100.0 ± 0.0^{x}	73.0 ± 1.6^{yz}	1.5 ± 0.1^{xy}
100% K^+	(SG100)	20	4.1 ± 0.1^{x}	14.8 ± 0.6^{x}	4.6 ± 0.2^{z}	64.5 ± 4.2^{x}	100.0 ± 0.0^{x}	78.7 ± 2.6^{z}	1.2 ± 0.04^{x}
80% K^+	(SG80)	20	4.0 ± 0.2^{x}	13.9 ± 0.5^{x}	4.2 ± 0.2^{yz}	59.5 ± 1.5^{x}	100.0 ± 0.0^{x}	76.4 ± 2.6^{yz}	1.3 ± 0.03^{xy}
60% K^+	(SG60)	20	4.0 ± 0.1^{x}	14.0 ± 0.6^{x}	4.3 ± 0.2^{yz}	58.9 ± 2.4^{x}	95.8 ± 4.1^{x}	77.9 ± 2.9^{z}	1.3 ± 0.03^{xy}
40% K^+	(SG40)	20	3.9 ± 0.2^{x}	10.1 ± 0.8^{y}	2.8 ± 0.2^{x}	35.1 ± 4.8^{y}	91.7 ± 8.3^{x}	56.1 ± 4.2^{x}	1.7 ± 0.2^{y}
100% K^+	(SG100-30)	30	3.8 ± 0.1^{x}	12.3 ± 0.5^{x}	3.6 ± 0.2^{y}	51.1 ± 3.4^{x}	100.0 ± 0.0^{x}	66.3 ± 2.6^{y}	1.3 ± 0.1^{xy}

[a] Data are means ± S.E. for four replicate tanks. Means in each column with a different superscript are significantly different ($P < 0.05$).
[b] Adjusted wet weight gain = [final total weight + weight of mortalities] − [initial total weight + weight of replacement fish].
[c] Mean total feed consumed per tank expressed as grams dry weight.
[d] Food conversion ratio = weight of feed fed/adjusted wet weight fish gain.

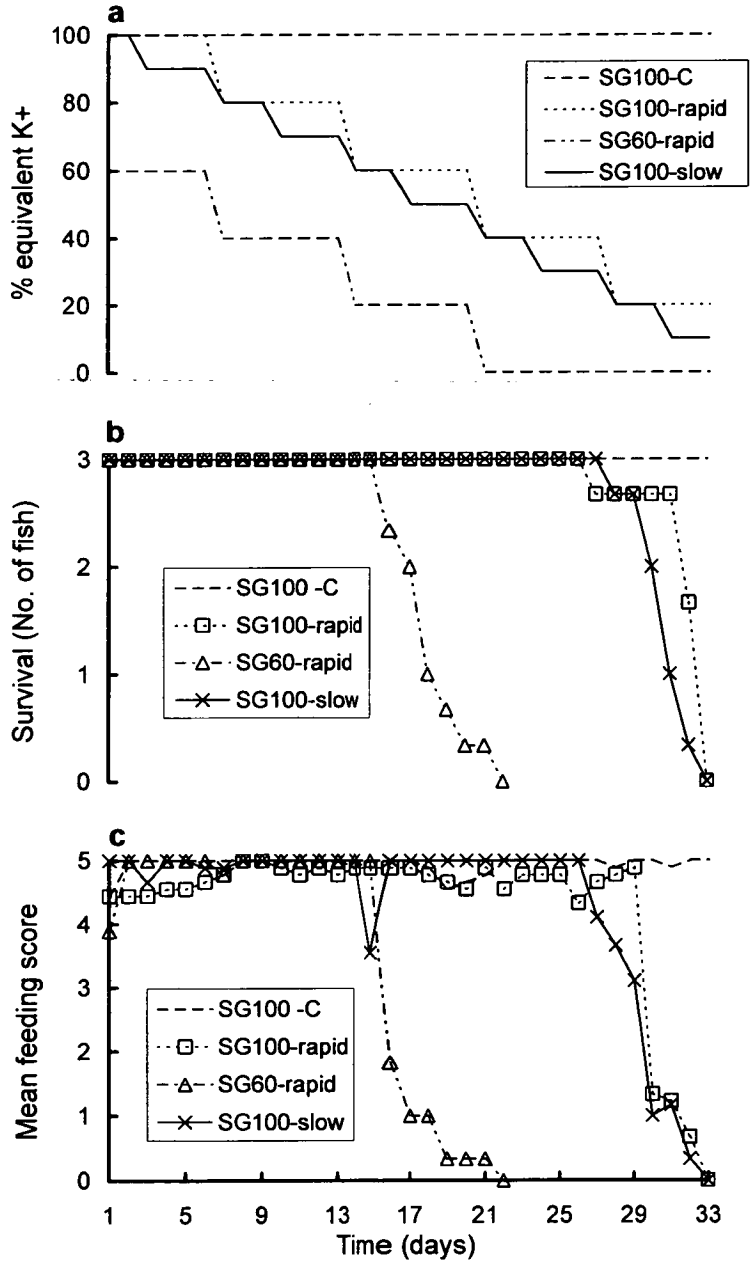

Fig. 1. Potassium dilution schedule (a), mean survival (b) and mean feeding score (c) of juvenile snapper *P. auratus* exposed to decreasing concentration of potassium in saline groundwater from Wakool. For survival, data are means of $n = 3$ buckets (3 fish/bucket). For feeding, fish were scored from 5 (normal) to 1(abnormal). (Experiment 4).

low and similar for fish grown in the CS, SG60, SG80, SG100 and SG100-30 treatments, but was higher for snapper grown in the SG40 treatment (Table 5).

3.4. Experiment 4: acclimation of juveniles to raw saline groundwater

All snapper survived and the feeding and swimming behaviour of the fish were similar and normal for all acclimation treatments provided the K^+ concentration was $\geq 40\%$ of that in equivalent salinity CS (Fig. 1). However, when the K^+ concentration was diluted to 20% for the SG100-slow, SG100-rapid and SG60-rapid treatments, some fish lost equilibrium and stopped feeding within 1–2 days and all fish were moribund after 4–6 days.

3.5. Experiment 5: effect of K^+ or Cl^-

Snapper that were held in the NaCl-fortified groundwater treatment began to lose equilibrium of buoyancy and appetite after 1 day following transfer from coastal seawater. After 2 days, four fish were moribund and the remaining fish were lethargic and did not feed well. This trend continued, and after 4 days all fish in the NaCl-fortified groundwater treatment were moribund. Snapper held in the KCl-fortified groundwater treatment for 4 days all survived, and were actively swimming and feeding during this time.

4. Discussion

Raw saline groundwater from Wakool was not suitable for survival and growth of juvenile snapper. Within 1–2 days following transfer of snapper from coastal seawater (CS) to raw saline groundwater (SG), fish started to lose equilibrium of buoyancy, floated upside down and did not feed well. The performance of snapper did not improve with time and by approximately 4 days after transfer all snapper were moribund or dead.

The concentration of the major ions, Cl^-, Na^+, SO_4^{2-}, Mg^{2+} and Ca^{2+} in SG was either similar to or greater than that of equivalent salinity CS. However, the concentration of K^+ in the SG was extremely low, being only 4.5% (9.2 mg l^{-1}; 19.6‰) of that available in equivalent salinity CS (203 mg l^{-1}). The salinity and chemistry of saline groundwater can vary widely. For example, in a similar study in Texas, USA saline groundwater was collected from 35 sites and representative samples evaluated for their suitability for aquaculture of red drum, *Sciaenops ocellatus* (Forsberg et al., 1996). The concentration of the major ions were: salinity, 2–35‰; Cl^-, 639–15,443 mg l^{-1}; Na^+, 537–9403 mg l^{-1}; SO_4^{2-}, 164–5934 mg l^{-1}; Mg^{2+}, 8–1263 mg l^{-1}; K^+, 5–87 mg l^{-1}; and Ca^{2+}, 36–1179 mg l^{-1}.

Sodium, potassium and chloride are essential minerals for animals due to their role in electrolyte and acid-base balance (Wilson and El Naggar, 1992). Potassium is the main cation of intracellular fluids while sodium and chloride are the major extracellular ions. Osmoregulation, or the maintenance of constant extracellular and intracellular osmolality, is mostly determined by the homeostasis of these ions (Teeter, 1997). Fish can

readily derive all or a portion of these minerals from the water by unidirectional diffusion across the gills and the gut or they can be obtained from food (Shearer, 1988; Lall, 1989; Wilson and El Naggar, 1992). Because fish can sequester potassium from the water, it has been difficult to conduct studies to determine dietary requirement of this ion without altering the ionic composition of the water (Wilson and El Naggar, 1992). Consequently, there is a paucity of information describing the quantitative requirement of potassium (Lall, 1989).

The estuarine Japanese red sea bream, the same species as snapper (Paulin, 1990), and the freshwater channel catfish did not require dietary potassium when grown in seawater and freshwater (4 mg l^{-1} K^+), respectively (Sakomoto and Yone, 1978; Wilson and El Naggar, 1992), thus indicating that these fish had obtained all necessary potassium from the water. Alternatively, Shearer (1988) showed that juvenile chinook salmon, *Oncorhynchus tshawytscha*, grown in freshwater with a potassium concentration of < 1 mg l^{-1}, were unable to sequester sufficient potassium from the water and required a dietary potassium concentration of 0.8% for maximum growth. Wilson and El Naggar (1992), however, contend that 0.8% is an overestimate of the potassium requirement for chinook salmon. Potassium-deficient chinook salmon initially displayed reduced growth rate due to anorexia and poor food conversion and ultimately fish died.

Results of our study showed that performance of snapper improved dramatically when KCl was added to the SG. The enhanced performance of fish was due to an increase in the concentration of potassium ions rather than an increase in the concentration of chloride ions (Experiment 5). In Experiment 3, growth, feed consumption and FCR after 42 days were the same for snapper held in CS and SG (both 20‰), provided the potassium concentration of the SG was fortified to 60–100% ($\sim$ 124–207 mg l^{-1} K^+) of the potassium concentration as occurred in equivalent salinity CS. Also, the performance of snapper grown in higher salinity SG (30‰) and fortified to provide 100% ($\sim$ 310 mg l^{-1} K^+) was the same as the CS control fish. However, snapper held in SG (20‰) fortified with 40% ($\sim$ 83 mg l^{-1} K^+) of the potassium concentration as occurred in equivalent salinity CS, weighed significantly less ($P < 0.05$) and consumed almost 30% less feed and had a higher FCR than other treatments. Survival of snapper was not affected by these concentrations of potassium in SG; however, below 40% fortification of SG with potassium, fish were moribund within 2–6 days (Experiments 2 and 4).

Fortification of culture water with salts has improved growth and/or survival of fish in several other studies; however, results have varied with salt and water type. Survival of red drum was markedly improved from 0 to 93% when the calcium and chloride concentrations of saline groundwater (3–4‰) were increased from 36 to 337 mg l^{-1}, and 639 to 1296 mg l^{-1}, respectively following addition of $CaCl_2$; whereas addition of NaCl to the saline groundwater only improved survival slightly (Forsberg et al., 1996). On the other hand, addition of NaCl to freshwater and diluted seawater (1‰) resulted in much better survival (> twofold increase) of red drum than when $CaCl_2$ was added (Thomas and Wolters, 1992; Stahl et al., 1995). Clearly, the efficacy of remediating water by adding salt is dependent on the source and chemical composition of the water.

The symptoms of reduced growth and feed conversion and/or death displayed by snapper in SG with low potassium concentrations are similar to those of chinook salmon

fed potassium-deficient diets (Shearer, 1988) and also for euryhaline species, such as red drum, which were cultured in hypotonic water (Gatlin et al., 1992). In water with low ionic concentration, it is likely that there is a substantial loss of ions from the fish to the water. Osmoregulation, therefore, requires increased expenditure of metabolic energy, which may result in reduced fish performance (Bryan et al., 1988). We did not investigate the effects of low potassium concentration in SG on blood osmolality of snapper; however, further research in this area is warranted.

Snapper grown in SG fortified with 40% of the potassium concentration as occurred in equivalent salinity CS continued feeding during the experiment and it is likely, therefore, that some potassium was being derived from the feed. We did not determine the potassium concentration in the feed, which was a high protein, fish meal-based diet. Fish meal is typically a poor source of potassium; however, soybean meal, which constituted 12% of the diet, contains in excess of 2% potassium (Lall, 1989). It is likely, therefore, that the diet contained at least 0.24% potassium. This diet concentration is similar to the potassium requirement level reported for the channel catfish, 0.26% (Wilson and El Naggar, 1992) and some terrestrial animals, such as rats, 0.23% (Bieri, 1977) and pigs, 0.26% (Jensen et al., 1961). Clearly, the potassium concentration in the diet fed in our study did not provide adequate compensation for maximum growth of snapper grown in SG fortified with 40% potassium.

In general, dietary salt (NaCl) supplementation has resulted in little or no improvement in growth and feed conversion of some diadramous species, such as Atlantic salmon grown in freshwater and seawater (Shaw et al., 1975), and rainbow trout, *Oncorhynchus mykiss*, grown in freshwater (MacLeod, 1978). However, weight gain and food conversion of the euryhaline red drum grown in freshwater was improved when the diet was supplemented with NaCl at 2% or with both NaCl and KCl at 2% each (Gatlin et al., 1992). In our saline groundwater where potassium is deficient, improvements in performance of snapper may be achieved by supplementing the diet with KCl. This should be investigated.

It was not possible to acclimatise juvenile snapper to raw SG by gradually diluting SG fortified initially with either 60% or 100% of the potassium concentration as occurred in equivalent salinity CS. Regardless of dilution schedule, all snapper became moribund or died when the potassium concentration in the SG (20‰) was diluted to 20% (~ 40 mg l^{-1}) of the potassium concentration as occurred in equivalent salinity CS. Clearly, snapper were unable to compensate for potassium concentration in the SG of approximately 40 mg l^{-1} or lower. There is very little information available on the physiological and morphological responses of teleosts, in particular, marine-adapted fish, to low external ion concentrations. Perry and Wood (1985) showed that calcium uptake was higher in trout, *Salmo gairdneri*, when they were held in water with low-calcium concentration, compared to when they were held in water with high-calcium concentration, and this was correlated with a proliferation of lamellar chloride cells. When Mozambique tilapia, *Oreochromis mossambicus*, were held in magnesium-deficient freshwater, magnesium deficiency in the fish coincided with increased calcium and sodium content and a low potassium content of the body. An increase in opercula chloride cell density was found also in magnesium-deficient water (Bijvelds et al., 1997). Although there was no apparent difference in the performance of snapper grown

in SG fortified with 60–100% of the potassium concentration as occurred in equivalent salinity CS, morphological and physiological changes may have occurred, particularly in response to lower potassium concentration in this range. Further research is warranted to determine if snapper compensate physically to SG that is partially deficient in potassium and also if different (e.g. longer) dilution schedules allow snapper to acclimatise to raw SG.

Our results suggest that for normal snapper performance and osmoregulation, SG (20‰) from Wakool must be fortified with potassium to provide a minimum concentration of approximately 120 mg l^{-1}. Because salinity of the groundwater can vary and the interactions between ions during osmoregulation are complex, it may be more important to consider the ratio of ions rather than the specific concentration of individual ions in the water. Forsberg et al. (1996) suggested that the survival of red drum grown in saline groundwater was correlated with K^+/Cl^- and Na^+/K^+ ratios; however, the correlations resulted from within-treatment variation where the highest (100%) and lowest (70%) survival occurred in replicates of the same groundwater treatment. Regardless of this, mean survival was high (85–100%) and instantaneous growth was the same for red drum grown in four different saline groundwaters, which were not fortified with potassium, suggesting that potassium was not deficient. In these authors' experiments, the K^+/Cl^- ratios of saline groundwater (15‰) and the artificial seawater control ranged from 0.007 to 0.014, and 0.022, respectively. In our study, the K^+/Cl^- ratios of SG treatment water ranged from 0.001 for raw SG to 0.018 for 100% fortified SG. Survival and growth of snapper was achieved in SG provided the K^+/Cl^- ratio was greater than 0.007; however, maximum growth was achieved when the K^+/Cl^- ratio was greater than 0.01. When the K^+/Cl^- ratio was less than 0.007, snapper died.

5. Conclusion

Saline groundwater from Wakool was suitable for the culture of snapper in laboratory experiments provided the potassium concentration was fortified to supply a K^+/Cl^- ratio of 0.007–0.018. This was achieved easily by adding KCl to the saline groundwater. Following these results, a pilot-scale project has been established to evaluate the suitability of the saline groundwater for culture of snapper in ponds at Wakool. Further research should also investigate the potential to enhance growth of snapper in partially potassium-deficient saline groundwater by adding potassium to the diet. Research should also be conducted to determine if snapper change morphologically and/or physiologically in response to suboptimal concentration of potassium in the saline groundwater.

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Effect of photoperiod on growth and survival of snapper *Pagrus auratus* larvae

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Abstract

Experiments were done in 100-l recirculation tanks to determine the effects of photoperiod on (1) first-feeding and (2) post-swimbladder inflated snapper, *Pagrus auratus*, larvae. In Experiment 1, feeding onset, growth, initial swimbladder inflation, and tail flexion were assessed at five photoperiod treatments (0L:24D, 6L:18D, 12L:12D, 18L:6D, and 24L:0D) in larvae from 3 to 15 days after hatching (dah). Growth and development of first-feeding larvae increased with increasing photoperiod duration in the 12L:12D to 24L:0D treatments. Larvae did not start feeding in 0L:24D and onset of feeding was delayed by up to 3 days in 6L:18D. All larvae held in 0L:24D and 6L:18D died within 6 or 9 dah, respectively. Initial swimbladder inflation was best (80–100%) in an intermediate photoperiod of 12L:12D at 9 dah. By 15 dah, although the percentage of larvae with inflated swimbladders had increased in all treatments, swimbladder inflation in 12L:12D was 1.3 and 2.0 times greater than that of larvae in 18L:6D and 24L:0D, respectively. In the second experiment, growth and survival of snapper after the initial swimbladder inflation period (11–32 dah) were assessed at three photoperiod treatments (12L:12D, 18L:6D, and 24L:0D). Growth was greatest in 18L:6D in which wet weights (16.3±0.5 mg; mean±S.E.) and dry weights (2.8±0.1 mg; mean±S.E.) of larvae were approximately 1.3 and 1.9 times heavier than the larvae held in 24L:0D and 12L:12D, respectively. Survival of snapper larvae to 32 dah was not significantly different between the three photoperiod treatments, but power of the experiment to detect effects on survival was small due to large variability within treatments. Further research is needed to determine optimal photoperiods for the survival of the snapper larvae. Because of the potential for large larval mortality, if initial swimbladder inflation is not achieved, the optimal photoperiod for the period from feeding onset to swimbladder inflation (3–15

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dah) was deemed to be 12L:12D, whereas on the basis of growth parameters that were measured (total length, wet and dry weights), 18L:6D was determined to be the optimal photoperiod for the culture of snapper from the post-swimbladder window to metamorphosis (11–32 dah).

Keywords: Snapper; *Pagrus auratus*; Photoperiod; Swimbladder inflation; Larval growth; Larval survival

1. Introduction

Determination of the optimal environmental conditions for larvae and juvenile fish is necessary to maximise the production in hatcheries (Hart et al., 1996). One of the most important physical parameters for the growth and survival of fish larvae is photoperiod (Barnabe, 1990; Chatain and Ounais-Guschmann, 1991; Battaglene, 1995; Hart et al., 1996; Boeuf and Le Bail, 1999). Many marine fish larvae are visual predators and therefore require light for efficient planktivory (Blaxter, 1980; Boeuf and Le Bail, 1999). However, optimal photoperiod for larval development and growth and survival may differ, and also change with larval ontogeny.

In general, long photoperiod improves the performance of fish larvae, probably because of increased food availability (Boeuf and Le Bail, 1999). For example, photoperiods longer than that of ambient conditions increased growth of larval rabbitfish, *Siganus guttatus* (Duray and Kohno, 1988), sea bass, *Dicentrarchus labrax* (Barahona-Fernandes, 1979; Ronzani Cerqueira and Chatain, 1991), barramundi, *Lates calcarifer* (Barlow et al., 1995), and greenback flounder, *Rhombosolea tapirina* (Hart et al., 1996). Conversely, survival of larvae can be reduced in extended photoperiods (Barahona-Fernandes, 1979; Ronzani Cerqueira and Chatain, 1991). Decreased survival and increased prevalence of the deformities of fish larvae can be associated with the failure of larvae to inflate their swimbladders (Chatain and Dewavrin, 1989; Battaglene, 1995). Light intensity and photoperiod can both influence the ability for larvae to inflate their swimbladders, although this varies with species (Battaglene and Talbot, 1990; Ronzani Cerqueira and Chatain, 1991; Chatain and Ounais-Guschemann, 1990; Battaglene, 1995).

Snapper, *Pagrus auratus*, is an important commercial and recreational species found in the Australian and New Zealand waters (Bell et al., 1991; Battaglene and Bell, 1991; Pankhurst et al., 1991). However, wild catches are declining in Australia (ABARE, 1998), but aquaculture of snapper is increasing using intensive larval rearing techniques followed by growout in sea cages (Battaglene and Talbot, 1992; Battaglene and Fielder, 1997). These techniques are similar to those used for culture of the closely related Japanese red sea bream, *P. major* (Foscarini, 1988; Tabata and Taniguchi, 2000).

The environmental conditions in which Australian snapper larvae are typically reared are principally based on ambient conditions during the natural spring spawning season from August to November (Battaglene and Talbot, 1992). Photoperiod during this time ranges from approximately 10 to 14 h. However, the photoperiod for optimal development, growth, and survival of snapper larvae is unknown.

The aim of this study was to investigate the effects of photoperiod on larval snapper ontogeny to determine the optimal photoperiod protocol for swimbladder inflation, growth, and survival of snapper larvae reared in the tanks.

2. Materials and methods

Two laboratory experiments were done at the NSW Fisheries, Port Stephens Fisheries Centre from June to July 1999.

2.1. Source of larvae

Fertilised snapper eggs were obtained from first generation hatchery-reared (G1) broodstock held in a 17,000-l flat-bottomed tank, with an independent, recirculating system comprising an external 700-l egg collection sump, pump, and mechanical and biological filters. The tank was housed in a temperature- and photoperiod-controlled room.

Two groups of snapper larvae (groups 1 and 2) were produced on independent occasions after each of a 2.3 and 2.2 kg female snapper was induced to spawn using intramuscular pellet implants containing 207 and 120 μg/kg LHRHa (des-gly^{10}, D-trp^{6}, pro^{9}-ethylamide; Peptech Animal Health, Australia), respectively. Fertilised eggs were collected from the collection sump and were transferred to 2000-l conical-bottomed tanks with black sides and a white bottom filled with sterilised seawater (20 mg l^{-1} sodium hypochlorite for 24 h then neutralised with sodium thiosulphate) for incubation under darkness, recirculation was at 5 l min^{-1}, pH 7.5; dissolved oxygen (DO_2), 7.5 mg l^{-1}; temperature 19.3–20 °C, and salinity 35.3 ‰.

Hatching occurred approximately 30 h after fertilisation in both groups of larvae. Yolk sac larvae from group 1 were held in the incubation tank until 2 days after hatching (dah) under a 14L:10D (L:D, hours of light/dark) photoperiod provided by a fluorescent light (Philips White TLA 40W 33QS) at temperature range of 20.4–21.0 °C and slight aeration (200 ml min^{-1}).

Larvae from group 2 were held in the 2000-l incubation tank until 11 dah at a photoperiod of 12L:12D. Rotifers, *Brachionus plicatilis* enriched with the micro-algae *Pavlova lutheri* and Tahitian *Isochrysis* aff. *galbana*, and DHA Super Selco (Inve Aquaculture, Belgium) were fed to the larvae twice daily from the day of feeding onset (4 dah) at a density of 10 ml^{-1}. A surface skimmer was installed 5 dah to remove surface films and thus facilitate swimbladder inflation (Chatain and Ounais-Guschemann, 1990; Battaglene and Talbot, 1994). From 8 to 11 dah, approximately 25% of the tank was drained daily and refilled with fresh groundwater (0.6 ‰) to gradually reduce the salinity from 35 ‰ to 20 ‰. Previous research demonstrated that swimbladder inflation and survival of snapper larvae were not affected by a reduction in salinity, but growth was increased when salinity was reduced from 35 ‰ to 20 ‰ (Fielder, unpublished data).

2.2. Tanks used for the photoperiod experiments

Experiments were done in 100-l tanks as described by Fielder and Bardsley (1999). Surface skimmers were provided to each tank to remove surface films. Incandescent lighting (Osram Halogen Decostar 51 12 V 50 W) was provided overhead. Each tank was enclosed within a box made from black plastic sheeting to prevent the escape of light to the surrounding tanks. Water temperature range between tanks was maintained within 1 °C.

Approximately 5% of the water in each tank was changed each day and salinity was maintained by adding rainwater when needed.

2.3. Experiment 1: Effect of photoperiod from 3 to 15 dah

The aim of the experiment was to determine the effect of photoperiod on growth and development of first-feeding snapper larvae from 3 to 15 dah. The photoperiod treatments were 0L:24D, 6L:18D, 12L:12D, 18L:6D, and 24L:0D.

Larvae from group 1 (3 dah; 2.56±0.23 mm TL, n=10) were harvested from the 2000-l tank then transferred to a 50-l tank in which they were homogeneously mixed by slowly raising and lowering a perforated 27-cm diameter plastic disc. Randomly selected 200 ml samples of larvae were then transferred into each of the 30 100-l tanks. There were six randomly selected replicate tanks per photoperiod treatment. Physical tank parameters were maintained: pH range, 7.6–7.8; DO_2 range, 7.4–7.6 mg l^{-1}; temperature range, 20.4–21.0 °C; salinity range, 34.7–35.3‰; light intensity, 13.6±3.1 μmol s^{-1} m^{-2} (mean±S.D., n=30). Five additional 200 ml samples of larvae were taken to estimate initial stocking densities (1137±80 larvae $tank^{-1}$; mean±S.D.).

After stocking, and in accordance with the previously described reduction of salinity for the group 2 larvae, salinity was progressively reduced from 35.1±0.3‰ (n=30) to approximately 20‰ over 5 days by adding rainwater.

2.4. Experiment 2: Effect of photoperiod from post-swimbladder inflation

The aim of the experiment was to determine the effect of photoperiod on growth and development of post-swimbladder inflated snapper larvae from 11 to 32 dah. The photoperiod treatments were 12L:12D, 18L:6D, and 24L:0D.

Larvae from group 2 (11 dah) with inflated swimbladders (95% inflated, 4.63±0.36 mm TL, mean±S.D., n=20) were drained from the 2000-l tank, homogeneously mixed in a 20-l tank, and 200-ml randomly selected aliquots of larvae were transferred into each of the 24 100-l tanks. There were eight randomly selected replicate tanks per photoperiod treatment. Physical parameters were maintained: pH range, 7.6–7.8; DO_2 range, 8.7–8.8 mg l^{-1}; temperature range, 18.8–19.5 °C; salinity range, 19.2–20.7‰; light intensity, 13.3±2.4 μmol s^{-1} m^{-2} (mean±S.D., n=24). Stocking density was determined in a further five 200 ml randomly selected aliquots of larvae (529±83 larvae $tank^{-1}$; mean±S.D.).

2.5. Larvae feeding and sampling

Larvae in Experiments 1 and 2 were fed with enriched rotifers only (as described for rearing of group 2 larvae to 11 dah) at 0900 and 1500 h each day to maintain a density of approximately 10 rotifers ml^{-1}.

In both experiments, a randomly selected sample of 10 larvae was collected from each tank at approximately 1300 h every 3 days, and live larvae were observed under a dissecting microscope fitted with an ocular micrometer to determine measurement of TL (distance from the tip of the lower jaw to tip of the caudal fin), presence/absence of swimbladders, food in the gut, and development of tail flexion.

In Experiment 2, the final wet and dry weights of larvae were also measured in a randomly selected group of 10 larvae from each tank. Larvae were dried on blotting paper, placed onto a single, pre-weighed glass microscope slide, and larvae weight was determined to the nearest 0.01 mg with an analytical balance ("Analytical Plus", Ohaus, Switzerland). The slides were then placed into a drying oven at 106 °C for 16 h, after which time each slide was weighed to the nearest 0.01 mg to estimate the final larvae dry weight.

2.6. Daily water measurement

In the experiments, salinity, temperature, pH, and DO_2 were measured daily to the nearest 0.1 ‰, 0.1 °C, 0.1 pH unit, and 0.1 mg l^{-1}, respectively, using a water quality meter (Horiba U-10, Horiba, Japan). Total ammonia (Experiment 1 <0.6 mg l^{-1}; Experiment 2 <0.4 mg l^{-1}) was measured daily in at least one replicate tank from each treatment with a rapid test kit (E. Merck, Model 1.08024, Germany). Light intensity was measured to the nearest 0.1 μmol s^{-1} m^{-2} with a light meter (LI-COR, model Li-1776, USA).

2.7. Statistical analyses

Data were assessed for homogeneity of variance using Cochran's test (C; Winer et al., 1991). For Experiment 1, data for final total length (P<0.00001, C=0.9) were heteroge-

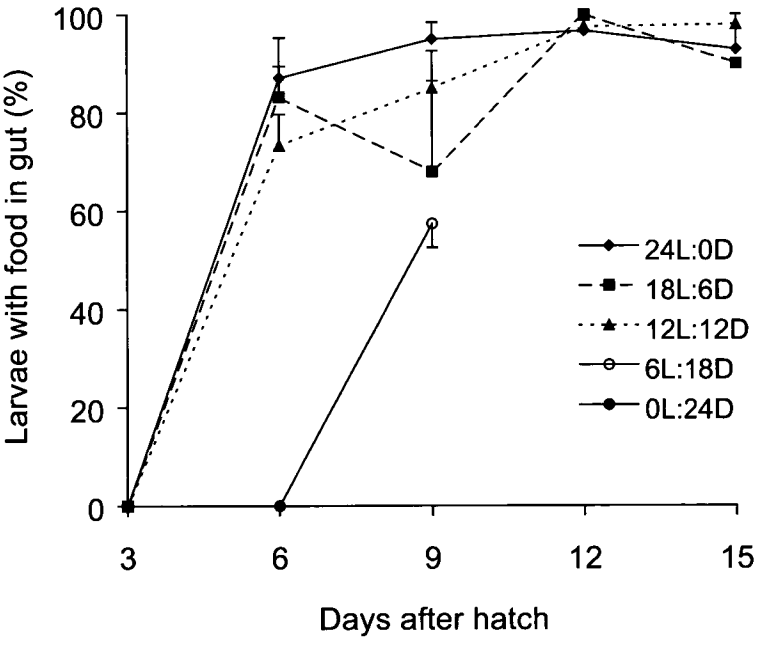

Fig. 1. Percentage of snapper larvae with rotifers in their gut grown from 3 to 15 dah in 100-l tanks under different photoperiod regimes in Experiment 1. Data are means±S.E.

neous and could not be transformed to satisfy the assumption of the homogeneity of variance. For Experiment 2, data for survival were transformed by Log_{10} to satisfy the assumption of homogeneity. Experiments were designed for analysis using single-factor analysis of variance (ANOVA). Where significant differences were found, means were compared by the Student–Newman–Keuls test (SNK). Data for final survival and mean final wet weight of larvae in Experiment 2 were also compared by regression analysis. A posteriori power analyses of ANOVA of mean final TL and final survival of snapper larvae in Experiment 2 were done to determine experimental power and number of replicates required to detect a range of minimum differences between means (Searcy-Bernal, 1994). Statistical analyses were conducted using Statgraphics Version 5.0 (STSC, USA).

3. Results

3.1. Experiment 1: Effect of photoperiod from 3 to 15 dah

Unexplained mortality of larvae occurred in four of the six replicate tanks in the 18L:6D treatment soon after stocking. This resulted in low numbers of surviving larvae in four replicate tanks, and by day 9, all larvae in these tanks had either died or were sampled.

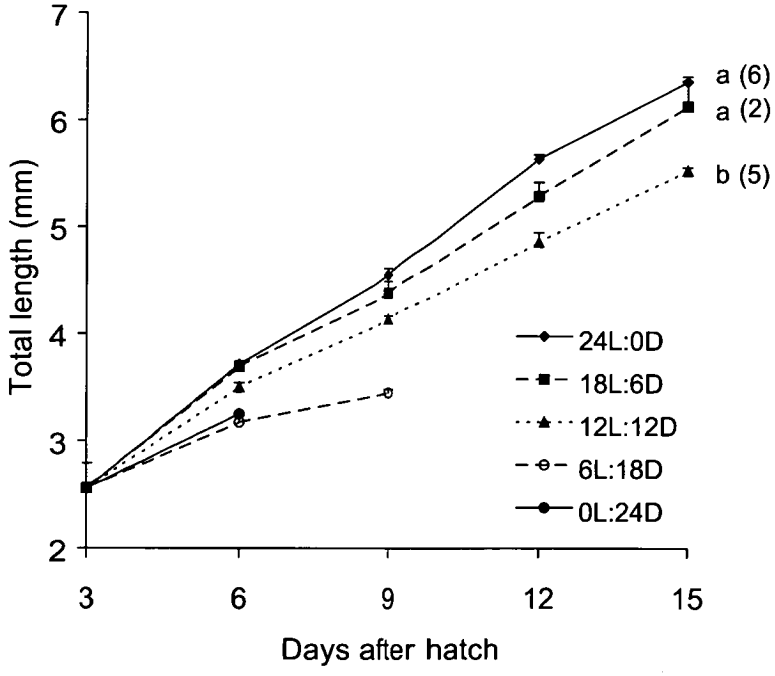

Fig. 2. Total length of snapper larvae grown from 3 to 15 dah in 100-l tanks under different photoperiod regimes in Experiment 1. Data are means±S.E. Points with different letters are significantly different ($P<0.05$); (n)=number of tanks sampled.

Snapper larvae that were held in total darkness did not start feeding (Fig. 1) and were all dead 3–4 days after transfer (6–7 dah). Rotifers were not observed in the gut of sampled larvae held in 6L:18D until 9 dah, but larvae held in 12L:12D, 18L:6D, and 24L:0D had begun feeding by 6 dah and the number of larvae with rotifers in the gut was high and similar ($P>0.05$) in these treatments (Fig. 1). Growth of larvae held in 6L:18D was slow (Fig. 2) and all larvae had died by 11 dah. Larvae held in 12L:12D, 18L:6D, and 24L:0D continued growing for the duration of the experiment. However, at 15 dah, the TL of larvae held in 18L:6D and 24L:0D was similar ($P>0.05$) and significantly greater ($P<0.05$) than that of larvae held in 12L:12D.

The development of tail flexion was significantly affected by photoperiod ($P<0.05$) (Fig. 3). Almost all (95%) the larvae that were sampled from the 24L:0D treatment had commenced tail flexion by 12 dah (mean TL±S.E.; 5.6±0.04 mm). Alternatively, no larvae in the 12L:12D treatment (mean TL±S.E.; 4.9±0.09 mm) and only 25% of larvae held in the 18L:6D treatment (mean TL±S.E.; 5.3±0.1 mm) had commenced tail flexion by this time. By 15 dah, 95% and 48% of larvae had commenced tail flexion in the 18L:6D and 12L:12D treatments, respectively (Fig. 3).

The number of larvae in the 12L:12D treatment with inflated swimbladders was significantly greater ($P<0.05$) than all the other treatments, which did not differ ($P>0.05$) (Fig. 4). At 9 dah, almost 90% of the larvae held in 12L:12D had inflated swimbladders, which was approximately two, three, and six times greater than the larvae held in the

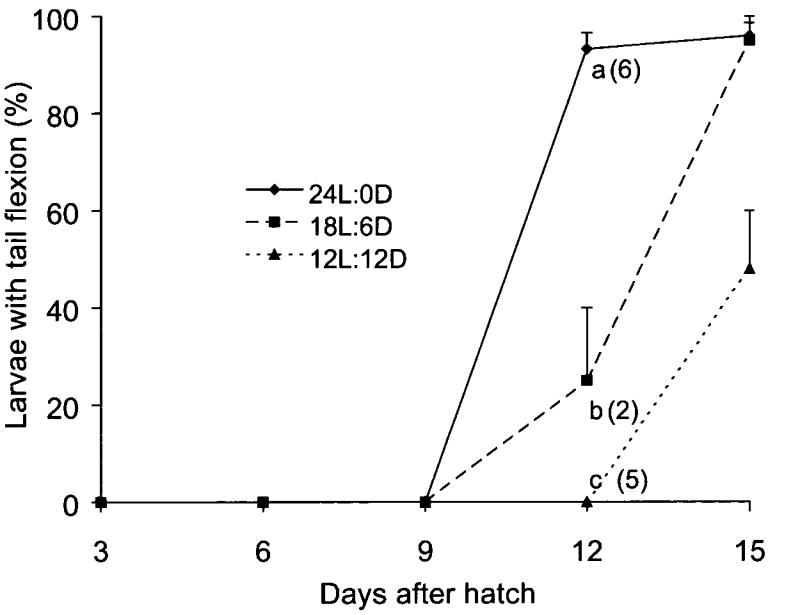

Fig. 3. Percentage of snapper larvae with tail flexion grown from 3 to 15 dah in 100-l tanks under different photoperiod regimes in Experiment 1. Data are means±S.E. Points with different letters are significantly different ($P<0.05$); (n)=number of tanks sampled.

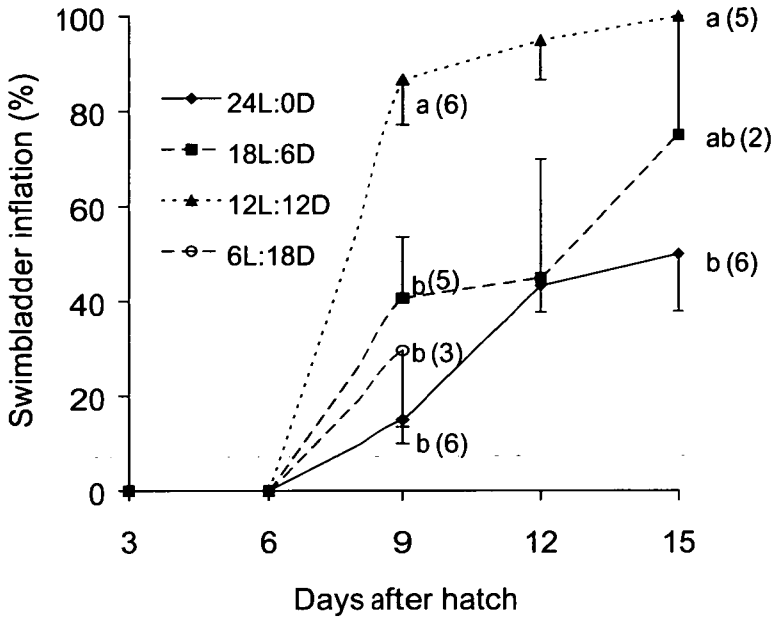

Fig. 4. Percentage of snapper larvae with inflated swimbladders grown from 3 to 15 dah in 100-l tanks under different photoperiod regimes in Experiment 1. Data are means±S.E. Points with different letters are significantly different ($P<0.05$); (n)=number of tanks sampled.

18L:6D, 6L:18D, and 24L:0D treatments, respectively. At 15 dah, the number of larvae that were sampled with inflated swimbladders had increased in the 12L:12D, 18L:6D, and 24L:0D treatments, but multiple comparisons of means did not clearly identify the differences due to insufficient replication (Fig. 4). Nevertheless, swimbladder inflation of larvae held in 12L:12D was 1.3 and 2.0 times greater than the 18L:6D and 24L:0D treatments.

3.2. Experiment 2: Effect of photoperiod from post-swimbladder inflation

At day 6 of the experiment (17 dah), larvae in one replicate tank of the 24L:0D treatment had abnormally distended (overinflated) swimbladders and began dying, and larvae in one replicate tank of the 18L:6D treatment were washed out of the tank onto the mechanical filter due to failure of the internal mesh screen. Data from both tanks were discarded from the experimental analysis.

Snapper larvae grew in all photoperiod treatments (Fig. 5). However, after 6 days (17 dah), the TL of larvae held in the 18L:6D and 24L:0D treatments was significantly greater ($P<0.05$) than that of larvae in the 12L:12D treatment. This trend continued and 32 dah larvae held in 18L:6D and 24L:0D, which did not differ significantly ($P>0.05$), were approximately 18.3% and 15.0% longer, respectively, than larvae in 12L:12D (Fig. 5).

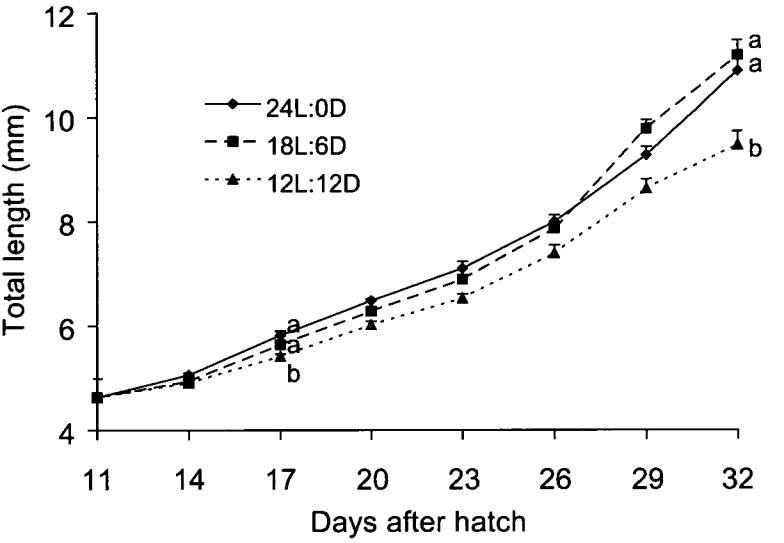

Fig. 5. Total length of snapper larvae grown from 11 to 32 dah in 100-l tanks under different photoperiod regimes in Experiment 2. Data are means±S.E. (n=8 for day 3; n=7 from day 6 onwards). Points with different letters are significantly different (P<0.05).

After 21 days (32 dah), the final wet and dry weights of larvae were significantly affected by photoperiod (P<0.05), with the heaviest larvae in the order of magnitude 18L:6D>24L:0D>12L:12D (Table 1). Larvae held in 18L:6D were approximately 1.3 and 1.9 times heavier than larvae held in 24L:0D and 12L:12D, respectively.

The ability to detect treatment differences varied greatly between the measured parameters of total length and wet or dry weights of snapper larvae. Significant differences between final mean wet weight (F=39.13, P<0.05) and dry weight (F=22.21, P<0.05) of snapper larvae were clearly identified, where 18L:6D>24L:0D>12L:12D. Analysis of mean data for final total length of snapper larvae, however, did not provide the same degree of sensitivity with n=7 replicates. It was possible to detect a difference of 1.72 mm in mean final total length of larvae (F=12.26, P<0.05) between treatments (18L:6D and 12L:12D) with a large degree of experimental power (0.97), but it was not possible to

Table 1
Final harvest survival, and wet and dry weights of snapper larvae grown from 11 to 32 dah in 100-l tanks under different photoperiod regimes in Experiment 2

Photoperiod regime	Survival (%)	Final wet weight (mg)	Final dry weight (mg)
12L:12D	16.6±6.9 a	8.48±0.7 5c	1.53±0.18 c
18L:6D	11.3±2.1 a	16.26±0.53 a	2.76±0.1 a
24L:0D	14.8±3.0 a	12.63±0.56 b	2.12±0.1 b

Data are means±S.E. for n=7 tanks. Means within columns with different letters are significantly different (P<0.05).

detect a significant difference ($P<0.05$) of 0.29 mm, which occurred between 18L:6D and 24L:0D treatments at the final harvest. Power analysis showed that approximately 90 replicate tanks were required to detect a difference of 0.29 mm between the largest and smallest means with a power of 0.8 at $P=0.05$ (Searcy-Bernal, 1994).

There was no difference in the survival of larvae between photoperiod treatments ($P>0.05$) (Table 1), but statistical power analysis on ANOVA of the number of surviving larvae showed that power of this experiment was low (0.1). Regression analysis showed that there was no significant relationship between the number of surviving larvae and the mean final wet weight of larvae ($F=1.64$, $P>0.05$). At each time of sampling, the number of larvae with rotifers in their gut did not differ ($P>0.05$) between photoperiod treatments and ranged from approximately 70% to 100%.

4. Discussion

Photoperiod clearly influenced the growth and development of snapper larvae and optimal photoperiod for culture of snapper varied with ontogeny. In Experiment 1, the snapper larvae held in constant darkness did not commence feeding and died within 3–4 days (6–7 dah) after the onset of the experiment. This confirms that snapper, like most marine teleosts, are visual feeders requiring light (Blaxter, 1986; Pankhurst, 1994; Pankhurst and Hilder, 1998; Cox and Pankhurst, 2000), and confirms an earlier report of the time of starvation in this species when denied with food (Pankhurst et al., 1991).

A short light phase (6L:18D) retarded feeding onset in young larvae by 3 days compared to other treatments (12L:12D, 18L:6D, and 24L:0D). While temperature has a potent effect on the rate of development of fish larvae (Blaxter, 1969, 1988; Youson, 1988), it appears that development (in this case the onset of feeding) is dissociated from temperature control in the 6L:18D larvae compared to other treatments. That is, despite the effect of temperature, development was retarded. This supports earlier reports that physical factors, other than temperature, affect the rate of larval development (Blaxter, 1969; Tandler and Helps, 1985).

Growth (total length) of larvae, 3–15 dah, was significantly greater in longer light phases of 18L:6D and 24L:0D than the 12L:12D treatment. This very likely reflects the benefits of increased duration of the visual feeding period (Tandler and Helps, 1985; Howell et al., 1998; Boeuf and Le Bail, 1999). The commencement of larval growth is profoundly affected by the duration of the transition period from endogenous to exogenous feeding (Parra and Yúfera, 2000). Successful first-feeding is influenced by the density of feed and the ability of larvae to catch prey (Parra and Yúfera, 2000). The ability to catch prey is a learned behaviour, which may occur on the first day of feeding for species such as black sea bass, *Centropristis striata* (Tucker, 1988), or may take 2–3 days for gilthead seabream, *Sparus aurata* (Parra and Yúfera, 2000). Therefore, long light phases provide more opportunity for larvae to actively develop their feeding skills.

In addition, assimilation of ingested rotifers, or other live feeds, by fish larvae can occur rapidly (Lubzens et al., 1989). For instance, 12-day-old herring larvae at 15 °C cleared their guts within 4 h of ingesting feed (Blaxter, 1965, cited in Blaxter, 1969). Therefore, more ingestion/digestion events can occur in longer feeding periods than in short feeding

periods. Also, in contrast to the retarding effect of a short light phase (6L:18D) on the onset of larval feeding, a longer light phase increased larval growth, which in turn was associated with accelerated larval development (as defined by the developmental milestone of tail flexion). Consequently, 95% of 24L:0D larvae commenced tail flexion by day 9 (12 dah) compared with 95% and 48% of larvae having commenced tail flexion in the 18L:6D and 12L:12D treatments after 12 days (15 dah), respectively. Despite some intraspecific variation, most fish species must reach a certain size before they can metamorphose into a juvenile (Youson, 1988). Length and/or growth rate of bony fish larvae is one of the most important factors that determines the development and the onset of metamorphosis (Youson, 1988). In a study similar to ours, Tandler and Helps (1985) demonstrated that photoperiod had a positive effect on growth rate of *S. aurata* larvae. That is, larvae grown under an extended light phase (24L:0D) were much larger (more developed) than larvae of the same age grown in a short light phase (12L:12D).

Initial swimbladder inflation, on the other hand, occurred in a greater degree when light was provided at the shorter light phase of 12L:12D. Snapper larvae, like many other physoclistous marine fish larvae, inflate their swimbladders by gulping air from the surface when endogenous yolk sac reserves are depleted and exogenous feeding begins (Kitajima et al., 1981; Chatain and Ounais-Guschemann, 1990; Battaglene, 1995). Swimbladder inflation may continue for up to approximately 1 week after yolk sac depletion, as the pneumatic duct closes within this time (Hunter and Sanchez, 1976; Battaglene, 1995). Early stage snapper, Australian bass, *Macquaria novemaculeata*, and European sea bass, *D. labrax*, larvae, for example, are negatively phototactic and high light intensity can discourage larvae from swimming to the surface (Ronzani Cerqueira and Chatain, 1991; Battaglene, 1995). Therefore, conditions for optimal swimbladder inflation are a compromise between periods of darkness when larvae can swim to the surface and periods of light when larvae can actively feed.

Swimbladder inflation is necessary for the subsequent growth and survival of the cultured physoclistous larvae (Battaglene, 1995) and failure to inflate swimbladders can result in reduced larval growth (Battaglene and Talbot, 1990, 1992), spinal deformities, including lordosis and kyphosis (Takashima, 1978; Chatain, 1994; Kitajima et al., 1994; Trotter et al., 2001), and high mortality (Spectorova and Doroshev, 1976; Chatain, 1986, 1987; Chapman et al., 1988; Chatain and Dewavrin, 1989). Despite the potential benefits of increased growth in larvae 3–15 dah at the longer light/shorter dark phases of 24L:0D and 18L:6D, these are likely to have been offset by the potential benefits of large rates of swimbladder inflation at 12L:12D. The latter is supported by the observation that cultured fish without swimbladders fail to thrive (Battaglene, 1995), and, as a result, methods have been developed to remove these fish from culture (Chatain and Corrao, 1992). Accordingly, we suggest a photoperiod of 12L:12D to be optimal for the culture of snapper during this early period of development.

In Experiment 2, growth (total length) of post-swimbladder inflated snapper larvae (11 dah) in 18L:6D and 24L:0D did not differ, but both treatments had a greater total length than larvae from the 12L:12D treatment. The heaviest larvae at experiment termination were in the 18L:6D treatment. There was no difference in the survival of larvae between photoperiod treatments but statistical power of the experiment (Searcy-Bernal, 1994) was very small (0.1) as the large variability within treatments meant that replication was

inadequate. Care must therefore be taken to accept the null hypothesis that photoperiod did not affect the survival of snapper larvae, and further research is warranted to determine optimal photoperiod for survival.

In general, long photoperiod improves larval rearing quality due to the 'synergistic effect of food availability and light' (Boeuf and Le Bail, 1999); larvae, being sight feeders, are able to feed for longer periods. However, optimal photoperiods are species-specific and maximal hours of feeding is not necessarily the most important factor of optimal conditions (Blaxter, 1986). For example, long-term continuous photoperiods increased the growth of 20 dah larvae of greenback flounder, *R. tapirina* (18=24>12>6 h light), but photoperiod from 6 to 24 h did not affect survival (Hart et al., 1996). Fuchs (1978) found that growth of sole, *Solea solea*, larvae was greater at 18 and 24 h light than at 12 h light, but survival was not affected by photoperiod. Similarly, growth of 8–20 dah barramundi, *L. calcarifer*, larvae was better at extended photoperiods (16=24>8 h light), but survival was not affected by photoperiod (Barlow et al., 1995). For the sparid, *S. aurata*, growth of larvae was greatest in continuous light of approximately 1000–3500 lx, but survival was best at an intermediate photoperiod of 15 h light (Peguin, 1984, cited in Tandler, 1993; Tandler and Helps, 1985). Survival of *D. labrax* was also best in a 12-h photoperiod, but like snapper in this study, larval growth in an 18-h photoperiod was greater than in the 12- or 24-h photoperiods (Barahona-Fernandes, 1979).

Larvae are continuously active in daylight in rearing tanks and this is associated with the search for food (Blaxter, 1986). Therefore, a longer light phase provides longer feeding duration but also extends the duration of the foraging (searching) behaviour such that a greater weight gain at 18L:6D compared to 24L:0D may simply reflect the energy budget. Japanese red sea bream (*P. major*) larvae, a proposed subspecies of *P. auratus* (Tabata and Taniguchi, 2000), undergo diurnal fluctuation in their swimbladder volume (Kitajima et al., 1985, 1993). During the day, when larvae are active and expending energy (Blaxter, 1986), the volume of swimbladders is reduced. At night, when larvae are not actively swimming (conserving energy), the swimbladders are inflated to maintain neutral buoyancy (Kitajima et al., 1993). There is a paucity of information relating to diurnal change in the swimbladder volume of snapper larvae. However, Pankhurst et al. (1991) observed that snapper larvae become inactive during the dark phase, suggesting that changes in swimbladder volume may be similar to the Red Sea bream. Therefore, accrual of conserved energy during a low activity 'dark' phase (18L:6D) may exceed the benefit of an extended but active feeding duration (24L:0D).

In addition, fish larvae tend to increase consumption of feed as prey density (availability) increases (Kamler, 1992), but in culture conditions where prey density is maintained at artificially high levels, consumption rates can be so high and assimilation rates can be so low (through rapid gut evacuation time) that growth is reduced (Howell et al., 1998). In our study, prey densities ranged from 1 to 2 ml^{-1} in the morning prior to feeding to 10 ml^{-1} after feeding. This range of prey density is considered large, and indeed, Parra and Yúfera (2000) demonstrated that growth of 15 dah gilthead seabream (*S. aurata)* larvae was the same when fed with prey at 1 and 10 ml^{-1}. It is possible that continual feeding (24L:0D) of snapper larvae resulted in suboptimal assimilation and therefore reduced growth compared to that in 18L:6D where larvae did not feed during the 'dark' phase.

Alternatively, snapper larvae may have displayed a circadian feeding pattern. This was demonstrated for *D. labrax* larvae, which remained actively swimming but had a marked reduction in feeding activity from 0000 to 0800 h (Ronzani Cerqueira and Chatain, 1991). Snapper larvae in continuous light (24L:0D) may have remained active but did not feed for a period of time, thus expending energy swimming rather than for growth. Snapper larvae in the 18L:6D treatment had a dark phase from 0200 to 0800 h, which may have coincided with the natural nonfeeding rhythm.

Clearly, differences in wet and dry weights of 32 dah snapper larvae are larger compared to corresponding differences in total length of larvae. Snapper larvae undergo metamorphosis from about 25 to 35 dah (Battaglene and Talbot, 1992) and it is most likely that the biomass of fish increases due to the development of internal organs (Foscarini, 1988), vertebral column and caudal skeleton (Matsuoka, 1982), myotomal musculature (Matsuoka and Iwai, 1984; Pankhurst et al., 1991), fins (Fukuhara, 1976, cited in Foscarini, 1988; Pankhurst et al., 1991), and scales (Battaglene, 1992). Therefore, estimate of wet and/or dry weights of 32 dah larvae provides a more sensitive evaluation of treatment effect than comparison of larval total length.

5. Conclusion

Optimal photoperiod for growth and development of snapper larvae in culture varied with life stage. For first-feeding snapper larvae, although growth increased as the photoperiod was increased, swimbladder inflation was best at 12L:12D. Once larvae had inflated their swimbladders, growth of larvae was greatest at an 18L:6D photoperiod. Survival of post-swimbladder inflated snapper larvae was not significantly affected by photoperiod. However, because the power of the experiment to detect treatment effects on survival was very small, further research is warranted to determine the effects of photoperiod on survival of snapper larvae. The photoperiod regime for optimal swimbladder inflation and growth to metamorphosis was 12L:12D from first-feed to swimbladder inflation and 18L:6D for post-swimbladder inflation to metamorphosis.

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